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Sigma-1 receptor inhibition ameliorates neuropathic pain induced by nerve transection

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tal vez sea la hora decisiva,

tal vez sea la hora de beber

el vino de la melancolía.

Tal vez sea que todo es tan distinto,

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Tino Tovar

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RESUMEN

RESUMEN

La Asociación Internacional para el estudio del dolor (IASP, por sus siglas en inglés) define el dolor neuropático como un dolor causado por una lesión o enfermedad del sistema nervioso somatosensorial. El dolor neuropático tiene una etiología muy diversa incluyendo la neuropatía periférica producida por un daño nervioso. Afecta al 8% de la población y se espera que esta prevalencia aumente en el futuro (Colloca et al., 2017). A pesar de los enormes esfuerzos tanto a nivel preclínico como clínico, los tratamientos farmacológicos disponibles hasta la fecha para paliar el dolor neuropático tienen una eficacia muy limitada, por lo que resulta de vital importancia la búsqueda de nuevas herramientas farmacológicas para su adecuado tratamiento (Colloca et al., 2017).

El receptor sigma-1 es una proteína chaperona regulada por ligando que bajo situaciones de estrés celular migra desde el retículo endoplásmico a la membrana plasmática, modulando la actividad de varios canales iónicos y receptores acoplados a proteínas G con los que interacciona físicamente. El receptor sigma-1 es una diana muy prometedora para el tratamiento del dolor neuropático, dónde ha sido ampliamente estudiado a nivel preclínico (Sánchez-Fernández et al., 2017). Entre los antagonistas selectivos sigma-1, el que ha sido caracterizado en mayor profundidad es el S1RA (Vela et al., 2015). Este fármaco ha sido evaluado en ensayos clínicos de fase II en pacientes con dolor neuropático inducido por quimioterapia, con resultados prometedores (Bruna et al., 2018). Aunque ya ha sido descrito el papel de la inhibición del receptor sigma-1 en modelos preclínicos de dolor neuropático inducidos por ligadura o constricción de nervios periféricos (Romero et al., 2012; Espinosa-Juárez et al., 2017), se desconoce su papel en la neuropatía producida por la sección de un nervio periférico. Realizar este estudio es interesante pues durante las intervenciones quirúrgicas (como toracotomía o mastectomía) la sección de nervios resulta inevitable, y como consecuencia, un gran número de pacientes desarrollan dolor neuropático postquirúrgico (Borsook et al., 2013). Además, los patrones de las alteraciones sensoriales así como los cambios de expresión génicos y neuroplásticos difieren entre los modelos de ligadura/constricción de nervio y los de denervación (Casals-Diaz et al. 2009), lo que podría implicar que la respuesta farmacológica fuese diferente también.

Por ello, en esta Tesis hemos evaluado el papel del receptor sigma-1 en la neuropatía que se produce en el modelo de lesión de nervio compartido (SNI, por sus siglas en inglés Spared Nerve Injury). El SNI consiste en seccionar dos de las tres ramas del nervio ciático dejando la rama sural intacta (Decosterd y Woolf, 2000). Por otra parte, se sabe que el receptor sigma-1 modula la analgesia opioide endógena periférica producida por ciertas células inmunitarias en el foco inflamatorio, contribuyendo a controlar el dolor (Tejada et al., 2018). Sin embargo, se desconoce por completo si los receptores sigma-1 también modulan esta analgesia opioide endógena durante el dolor neuropático.

Los mecanismos implicados en el alivio del dolor neuropático producidos por el bloqueo del receptor sigma-1 han sido estudiados mayoritariamente a nivel del sistema nervioso central, donde este receptor modula la sensibilización central (Sánchez-Fernández et al., 2017), disminuye la liberación de citoquinas proinflamatorias (Zhu et al., 2015) y reduce la activación astrocitaria y microglial (Zhu et al., 2015). Sin embargo, se desconoce la contribución del receptor sigma-1 en el dolor neuropático a nivel periférico, a pesar de que el receptor sigma-1 se expresa mayoritariamente en las neuronas sensoriales de los ganglios de la raíz dorsal (DRG, de sus siglas en inglés dorsal root ganglia) (Sánchez-Fernández et al., 2017). La sección de un nervio periférico produce cambios en el sitio de la lesión así como en los somas de las neuronas axotomizadas. Por ejemplo, las neuronas lesionadas sufren un proceso de cromatolisis desplazando su núcleo desde una posición central hasta la periferia; así mismo estas neuronas comienzan a expresar factores de transcripción como el ATF3 (Chandran et al., 2016; Johnson and Sears, 2013) (de sus siglas en inglés activating transcription factor 3). Entre estos cambios, la infiltración de macrófagos, atraídos por la citoquina CCL2, parece que juega un papel clave en el desarrollo del dolor neuropático (Zigmond and Echevarria, 2019). La posible participación del receptor sigma-1 en este proceso neuroinflamatorio a nivel periférico se desconoce hasta la fecha.

Teniendo en cuenta todos estos antecedentes, en esta tesis doctoral nos centramos en el estudio del efecto de la inhibición del receptor sigma-1 sobre la hipersensibilidad dolorosa producida por el modelo SNI, así como de la contribución de la analgesia

opioide endógena a este efecto del receptor sigma-1. Además, también estudiamos la contribución del receptor sigma-1 a la infiltración de macrófagos dentro del DRG que se produce durante el SNI.

Inicialmente estudiamos el desarrollo de la alodinia al frio (mediante el test de la acetona), la alodinia mecánica (con el test de von Frey manual) y la hiperalgesia al calor (mediante el test de Hargreaves) en la neuropatía producida tras el SNI en ratones hembra tipo salvaje (WT, de sus siglas en inglés wild-type) y mutante desprovistos del receptor sigma-1 (Sig-1R-KO, de sus siglas en inglés knockout). El SNI produjo la misma hiperalgesia al calor en ratones WT y Sig-1R-KO. Sin embargo, la alodinia mecánica fue menor en ratones Sig-1R-KO que en ratones WT, y los ratones Sig-1R-KO no desarrollaron alodinia al frio. Después estudiamos el efecto de la inhibición farmacológica del receptor sigma-1 y para ello administramos el antagonista selectivo S1RA (8-128 mg/kg, s.c.). El S1RA inhibió las distintas alteraciones sensoriales producidas por el SNI en los ratones WT de manera dosis dependiente. De las tres modalidades sensoriales exploradas, la alodinia al frio fue la más sensible al efecto del S1RA, ya que fue revertida completamente con la dosis de 16 mg/kg, mientras que la dosis de 64 mg/kg fue necesaria para revertir la hiperalgesia al calor y la de 128 mg/kg para revertir parcialmente la alodinia mecánica. Los efectos antinociceptivos del S1RA fueron revertidos por completo por el agonista selectivo del receptor sigma-1 PRE-084 (32 mg/kg, s.c.), y estuvieron totalmente ausentes en los ratones Sig-1R-KO (64-128 mg/kg, s.c.), demostrando así la selectividad del S1RA por los receptores sigma-1. Por lo tanto, el receptor sigma-1 juega un papel importante en el dolor neuropático inducido por la sección del nervio ciático en ratón.

Para comprobar la contribución del sistema opioide endógeno al efecto antineuropático producido por el antagonismo sigma-1 tras la sección del nervio ciático, inyectamos naloxona o su derivado de acción periférica naloxona metiodida, previamente al S1RA. La administración de naloxona (1 mg/kg, s.c.) o naloxona-metiodida (2 mg/kg, s.c.), revirtió completamente el efecto del S1RA (16-128 mg/kg, s.c.) en la hiperalgesia al calor y la alodinia mecánica, pero no en la alodinia al frio, indicando que los efectos producidos por el antagonismo del receptor sigma-1 están mediados por mecanismos dependientes de opioides (en alodinia mecánica e

hiperalgesia al calor) y no dependientes de opioides (en alodinia al frio). La administración de morfina (0.5-2 mg/kg, s.c.) revirtió completamente la hipersensibilidad al calor y al frio y parcialmente la alodinia mecánica. La administración de naloxona revirtió el efecto de la morfina en las tres modalidades sensoriales exploradas, mientras que la naloxona-metiodida solo consiguió revertir la inhibición por morfina de la hiperalgesia al calor y la alodinia mecánica.

A continuación estudiamos el efecto del tratamiento repetido con una antagonista sigma-1 en la neuropatía producida tras el SNI. El tratamiento continuado con S1RA (25 mg/kg, i.p., 2 veces al día durante 10 días), redujo la hipersensibilidad mecánica, al frio y al calor producida por el SNI sin inducir tolerancia al efecto analgésico. Estos efectos antinociceptivos fueron observados hasta 12 horas después de la última administración del fármaco, cuando el S1RA a nivel plasmático y cerebral fue indetectable mediante HPLC-MS/MS; indicando, por tanto, que están mediados por un efecto farmacodinámico prolongado en el tiempo. En cambio, cuando se administró de forma aguda la misma dosis de S1RA (25 mg/kg, i.p.) solo fue capaz de revertir la alodinia al frio, debido a la mayor potencia que presenta el S1RA sobre esta manifestación comportamental, y el efecto fue de muy corta duración, habiendo desaparecido a los 90 min de su administración.

Además, también nos propusimos estudiar el mecanismo por el cual el receptor sigma-1 paliaba la hipersensibilidad dolorosa producida por el SNI a nivel periférico. Para ello, a los 7 días del SNI se extrajeron los DRGs donde están localizados mayoritariamente los cuerpos de las neuronas que reciben las aferencias del nervio ciático de animales WT y Sig-1R-KO y se realizó un estudio inmunohistoquímico con anticuerpos específicos para neuronas (NeuN), estrés celular (ATF3), macrófagos (Iba-1) y para el propio receptor sigma-1. Asimismo, se realizaron experimentos mediante citometría de flujo (FACs, de sus siglas en inglés Fluorescence-Activated Cell Sorting) con el marcador para macrófagos (CD11b). En los estudios inmunohistoquímicos encontramos que las neuronas lesionadas mostraron su núcleo de forma excéntrica, una reducción a la baja en el marcaje de NeuN, así como un cambio en el patrón de distribución celular del receptor sigma-1, que pasó de un marcaje celular homogéneo (excluido el núcleo celular), a una concentración del marcaje en la periferia del soma

neuronal y alrededor del núcleo celular. Además, el 78% de las neuronas que externalizaban el receptor sigma-1 expresaban ATF3; es decir las neuronas lesionadas externalizaron el receptor sigma-1. Para estudiar si el receptor sigma-1 tiene alguna relevancia en la infiltración de los macrófagos en el DRG tras el SNI, realizamos un ensayo de inmunohistoquímica con los anticuerpos dirigidos contra el receptor sigma-1 y contra los macrófagos. El número de neuronas que estaban rodeadas de macrófagos fue mayor en las neuronas con el receptor sigma-1 translocado (69.5%) que en aquellas donde el receptor sigma-1 se encontraba homogéneamente distribuido por el citoplasma (30.5%). Cabe destacar que estos macrófagos adoptaron una distribución alrededor de las neuronas dañadas formando estructuras en forma de anillo. Para aclarar el papel del receptor sigma-1 en la infiltración de macrófagos después del SNI, realizamos un ensayo de inmunohistoquímica en animales Sig-1R-KO. Se observó que el incremento del número de macrófagos era significativamente menor en animales mutantes desprovistos del receptor sigma-1 que en animales salvajes. Estos resultados se confirmaron mediante FACs, ya que comprobamos que el SNI produjo una infiltración masiva de macrófagos en los DRG de ratones WT, mientras que este reclutamiento fue mucho menor en animales Sig-1R-KO. Estos resultados podrían indicar que existe una posible interacción neurona-macrófago y que el receptor sigma-1 puede estar participando en el reclutamiento de estas células inmunitarias.

Por último, se sabe que tras una lesión de un nervio periférico las neuronas del DRG liberan la quimioquina CCL2 (también denominada MCP-1) que tiene un papel relevante en el reclutamiento de macrófagos hasta el DRG. Por tanto, nos propusimos estudiar el papel del receptor sigma-1 en la liberación de la quimioquina CCL2 en el DRG. Para ello, mediante ELISA, realizamos una determinación de los niveles de CCL2 después del SNI en ratones WT y Sig-1R-KO. Los DRGs de los animales Sig-1R-KO mostraron niveles más bajos de esta quimioquina en todos los puntos temporales estudiados (6 horas, 24 horas, 3 días y 7 días, tras el SNI) pero especialmente a día 3, que es donde se encontró el pico máximo de concentración de CCL2 en los animales WT. Estos resultados sugieren que el receptor sigma-1 podría modular la liberación de la quimioquina CCL2 en el DRG después del SNI y que esto podría contribuir a la menor

infiltración de macrófagos encontrada en los animales Sig-1R-KO tras el SNI y la consiguiente reducción de la hipersensibilidad dolorosa que mostraron los animales desprovistos del receptor sigma-1.

En conclusión, el receptor sigma-1 juega un papel relevante en la fisiopatología de la neuropatía inducida por sección del nervio ciático, reduciendo el proceso neuroinflamatorio dependiente de macrófagos que acontece durante dicha neuropatía en los DRGs e inhibiendo las manifestaciones dolorosas de la neuropatía, mediante mecanismos dependientes e independientes de opioides endógenos. Estos datos sugieren que el antagonismo del receptor sigma-1 podría ser una herramienta terapéutica potencial para prevenir o tratar el dolor neuropático inducido por sección de nervios periféricos.

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INTRODUCTION

INTRODUCTION

The International Association for the Study of Pain (IASP) defines neuropathic pain as "pain caused by a lesion or disease of the somatosensory nervous system", including peripheral nervous system (PNS) and central nervous system (CNS). Common conditions associated with neuropathic pain are of diverse etiology: toxic (e.g.: chemotherapy-induced neuropathy, alcoholic neuropathy), traumatic (e.g.: phantom limb pain, post-surgical/traumatic neuropathy), metabolic (e.g.: diabetic neuropathy, vitamin B12 deficiency), infectious (e.g.: post-herpetic neuralgia, HIV neuropathy), invasive/compressive (e.g.: painful radiculopathy, cancer), stroke (e.g.: central post-stroke pain), multiple sclerosis and hereditary diseases (e.g.: Charcot-Marie-Tooth disease, erythromelalgia) (Colloca et al., 2017; Alles and Smith., 2018). The prevalence of neuropathic pain in the general population has been estimated to be in the range of 6.9-10% (van Hecke et al., 2014), and it is expected to raise in the future (Colloca et al., 2017). This incidence is likely to increase owing to global population ageing, improved survival from cancer after chemotherapy and increased incidence of diabetes mellitus among other factors (Colloca et al., 2017).

Lesions or diseases of the somatosensory nervous system can lead to altered and disordered transmission of sensory signals into the spinal cord and the brain. Clinical manifestations of neuropathic pain are usually discussed in term of positive and negative signs and symptoms. Positive manifestations include pain, paresthesia and dysesthesia and reflect an enhanced level of excitability in the nervous system. In contrast, negative manifestations such as hypoesthesia and hypoalgesia are due to a reduced impulse conduction in the neural tissues (Baron and Tölle, 2008). Typical positive signs of neuropathic pain are allodynia (pain due to a stimulus that does not normally provoke pain), hyperalgesia (increased pain from a stimulus that normally provokes pain), spontaneous pain (electric-shock-like or shooting pain without any external stimulus), and, occasionally, causalgia or incessant burning pain (Alles and Smith, 2018). Sleep disturbances, anxiety and depression are frequent and severe in patients with neuropathic pain, and quality of life is more impaired in patients with chronic neuropathic pain than in those with chronic non-neuropathic pain (Colloca et al., 2017). Neuropathic pain is also seen in complex regional pain syndrome as a result

of neurogenic inflammation and/or pathologic cross talk between sensory and sympathetic nerves (Calvo et al., 2012; Alles and Smith, 2018).

Neuropathic pain syndromes can be divided into peripheral or central neuropathic pain according to the anatomical localization of the lesion or disease (Meacham et al., 2017). Central neuropathic pain usually results as a consequence of spinal cord injury, stroke, or multiple sclerosis (Costigan et al., 2009b). Cerebrovascular disease affecting the central somatosensory pathways (poststroke pain) and neurodegenerative diseases (notably Parkinson disease) are brain disorders that often cause central neuropathic pain (Borsook, 2012). After spinal cord injury, a potentiation of the response of neurons to non-noxious and noxious peripheral stimulation is produced due to a maladaptive process in the synaptic circuits in the dorsal horn of the spinal cord (Gwak and Hulsebosch, 2011).

Peripheral disorders that cause neuropathic pain predominantly involves the small unmyelinated C fibers and the myelinated A fibers, namely, the Aβ and Aδ fibers (Finnerup et al., 2016). Peripheral nerve damage can result in chronic neuropathic pain after being affected by metabolic damage, toxins, drugs, cytokines, and other inflammatory mediators (White et al., 2007). While the insult may be localized, the responses that lead to chronic pain can be spread out. Injuries throughout the axon such as transection, compression, hypoxia, inflammation, infection and chemical damage can induce fiber degeneration and changes in some protein expression (channels, receptors, etc) and lead to ectopic activity of primary sensory neurons resulting in peripheral neuropathic pain (Meacham et al., 2017). Peripheral sensitization in response to peripheral nerve injury may results in an increased primary afferent drive that can contribute to changes in the spinal cord and lead to central sensitization, which also contributes to neuropathic pain states (Costigan et al., 2009b; Meacham et al., 2017).

Despite the enormous effort made by both clinical and preclinical research, there are no well-established treatments to ameliorate neuropathic pain in all patients and therefore, it remains a clinically-relevant unmet need (Finnerup et al., 2015; Cavaletti and Marmiroli, 2018). Therefore, identification of new drug targets for neuropathic

pain treatment is an important goal. The sigma-1 receptor (Sig-1R) is a unique ligand-operated chaperone present in key areas for pain control, in both the peripheral and central nervous system. Sigma-1 receptors interact with a variety of protein targets (ion channels and receptors) to modulate their function (Sánchez-Fernández et al., 2017). Sigma-1 antagonists have been shown to exert antinociceptive effects in preclinical models of neuropathic pain induced by nerve trauma (Roh et al., 2008; De la Puente et al., 2009; Díaz et al., 2012; Romero et al., 2012; Bura et al., 2013; Choi et al., 2013; Gris et al., 2016; Kang et al., 2016), chemical injury by antineoplastic (Nieto et al., 2012; Nieto et al., 2014; Gris et al., 2016; Paniagua et al., 2019), spinal cord injury (Choi et al., 2016; Castany et al., 2018; Castany et al., 2019) and diabetic neuropathy (Paniagua et al., 2017; Wang et al., 2018).

S1RA (also named MR309 and E-52862) is a Sig-1R antagonist that completed Phase I clinical studies with good tolerability and kinetic results (Abadías et al., 2013). S1RA has also been evaluated recently in a Phase II clinical trial for chemotherapy-induced neuropathic pain showing promising results (Bruna et al., 2018; Bruna and Velasco, 2018), being the first Sig-1R ligand developed with an intended indication for pain treatment. Treatment with S1RA was able to reduce acute oxaliplatin-induced peripheral neuropathy and it led to a higher oxaliplatin exposure, showing a potential neuroprotective role for chronic cumulative oxaliplatin-induced peripheral neuropathy in patients with colorectal cancer (Bruna et al., 2018; Bruna and Velasco, 2018).

In addition, Sig-1R modulate opioid analgesia in nociceptive pain (Vidal Torres et al., 2013; Sánchez-Fernandez et al., 2013; Sánchez-Fernandez et al., 2014a), and control immune-driven peripheral endogenous opioid analgesia during inflammatory pain (Tejada et al., 2017). The endogenous opioidergic system plays a crucial role in the control of nociceptive responses at peripheral and central level (Stein, 2016). However, whether Sig-1R modulate opioid endogenous analgesia in neuropathic pain remains completely unknown.

Although the role of the Sig-1R inhibition in preclinical models of neuropathic pain induced by physical injury of a peripheral nerve has been described, those studies were limited to models of ligation/constriction of the sciatic nerve (Roh et al., 2008; de

la Puente et al., 2009; Romero et al., 2012). However, the patterns of pain-like behaviors (Casals-Diaz et al. 2009), gene profilings (Griffin et al., 2007; Costigan et al., 2010) and neuroplastic changes (Casals-Diaz et al. 2009), differ between models of constriction/ligation and models of denervation of the sciatic nerve, suggesting that the response to drug treatment might also differ. During surgical interventions nerve transections are inevitably, and as a consequence, a significant number of patients develop neuropathic pain (Borsook et al., 2013).

Taking into account these antecedents, the aim of this PhD project is to study the role of Sig-1R on neuropathic pain in a model of transection of the sciatic nerve. The model used, named spared nerve injury (SNI), consist in the transection of two of the three branches of the sciatic nerve and produces a persistent neuropathic pain in rodents, manifested by marked hypersensitivity in the territory of the intact branch (Decosterd and Woolf, 2000).

In this Introduction, we will briefly summarize in the first chapter the pathophysiological mechanisms associated to neuropathic pain induced by peripheral nerve injury and in the second chapter the contribution of sigma-1 receptors to this modality of neuropathic pain.

1. NEUROPATHIC PAIN

Neuropathic pain mechanisms have been extensively studied in different animal models. Although neuropathic pain can be originated by a lesion of the CNS or PNS, mechanisms underlying central neuropathic pain have been much less studied (Colloca et al., 2017); moreover, since the experimental model used in this PhD produces a lesion to PNS, this chapter will focus on peripheral neuropathic pain. Peripheral nerve injury mainly accounts for the beginning of neuropathic pain, as this peripheral lesion can initiate and maintain a sustained excitation of primary afferents. However, the primary neural damage is only the trigger of a cascade of multiple changes and alterations, distributed along the nervous system, such as ectopic generation of action potentials, loss of synaptic connectivity and formation of new synaptic circuits, facilitation and disinhibition of synaptic transmission, and neuroimmune interactions, that all together lead to and sustain neuropathic pain (Costigan et al., 2009b). These alterations change the nociceptive thresholds producing neuropathic pain-like behaviors (allodynia and hyperalgesia) in rodents. There are an ample number of animal models to study neuropathic pain and the behavioral patterns observed can vary with sex, age, species (generally, rat or mouse) and the own model of neuropathy (Kumar et al., 2018). There are models that involve the transection of a peripheral nerve and produce irreversible neuropathic pain, while other models, such as models of chemotherapy-induced peripheral neuropathy often induce reversible neuropathic pain.

1.1. Neurophysiological and neurochemical changes associated to neuropathic pain

Peripheral neuropathy alters the electrical properties of sensory nerves, which then leads to imbalances between central excitatory and inhibitory signaling such that inhibitory interneurons and descending control systems are impaired. At the peripheral and central nervous system, a gain of excitation and facilitation and a loss of inhibition are apparent, and in consequence, the sensory pathways shift to a state of

hyperexcitability, which over the time might contribute to the neuropathic pain state become chronic (Colloca et al., 2017).

After peripheral nerve injury, spontaneous activity can be generated at multiple places, apart from the neuroma (the place of injury with aborted axon growth), in the dorsal root ganglia (DRG) neurons, in neighboring intact afferents and in the nociceptive pathway within the central nervous system (Costigan et al., 2009b; Wang et al., 2011) (Figure A).

In rodents models of neuropathy, nerve section or constriction models, increased ectopic electrical discharge in myelinated axons (A fibers) begins generally within several hours of the induction of injury, and subsequently appears in unmyelinated axons (C fibers) within several days to weeks (Latremoliere and Woolf, 2009). Distinct classes of receptors and ion channels in specific sensory neuron subtypes have been implicated in the increased/sustained ectopic discharge. These receptors have at least three functions: transduction, conduction and modulation of synaptic transmission (Wang et al., 2011) (Figure A).

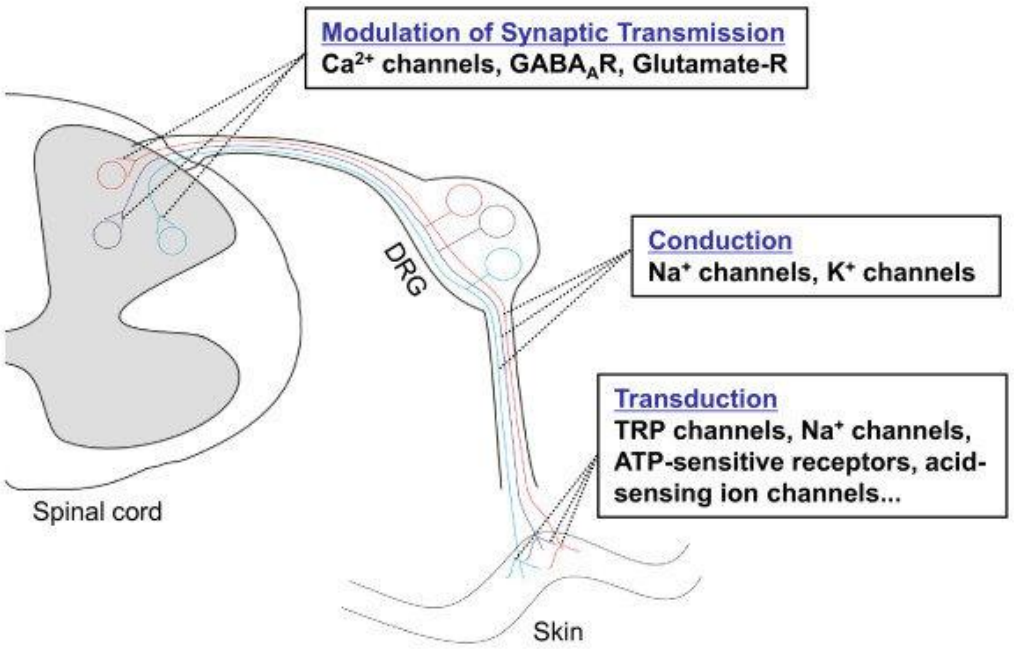

Figure A. Involvement of channels and receptors in the induction and modulation of pain. Different channels and receptors are involved in the transduction of noxious stimuli into electric impulses at the peripheral terminals of DRG neurons (TRP channels, Na^+ channels, ATP-sensitive receptors and acid sensing ion channels), in the conduction of action potentials along the axons (Na^+ and K^+ channels), and in the modulation of neurotransmitter release at presynaptic terminals of primary afferents in the dorsal horn (Ca^{2+} channels and GABA and glutamate receptors). *(Figure taken from Wang et al., 2011).*

The transient receptor potential (TRP) channels are ligand-gated ion channels that detect noxious pressure, extreme temperature and chemical stimuli, which are expressed in primary afferent nociceptors. There are different subtypes of TRPs involved in neuropathic pain, such as the transient receptor potential vanilloid 1 (TRPV1), the transient receptor potential ankyrin 1 (TRPA1) and the transient receptor potential melastatin 8 (TRPM8) (Basso and Altier, 2017). TRPV1 channel participates in both mechanical and heat-induced neuropathic pain and, whereas in the SNI model a downregulation of this channel has been described (Staaf et al., 2009), in the spinal nerve ligation (SNL) model there is an increase in TRPV1 expression (Marwaha et al., 2016). TRPA1 trigger peripheral neuropathies associated with diabetes, chemotherapy and mechanical nerve injury and participate in both mechanical and cold hypersensitivity (Marwaha et al., 2016; Basso and Altier, 2017). TRPM8 participates in the development of cold hypersensitivity caused by nerve trauma and antineoplastics, such as oxaliplatin, paclitaxel and vincritine (Basso and Altier, 2017). A downregulation of TRPA1 and TRPM8 is described while the transient receptor potential mucolipin 3 (TRPML3) expression is increased 4 days after SNI (Staaf et al., 2009).

Passive release of adenosine triphosphate (ATP) from severed nerve fibers and surrounding damaged tissue will activate type 2 purinergic receptors (P2R) expressed by sensory neurons and immune cells, but intact primary sensory neurons themselves also release ATP upon stimulation (Scholz and Woolf, 2007). The involvement of purinergic receptor P2X type 2 (P2X2), type 3 (P2X3) (Jarvis et al., 2002; Chen et al., 2005), type 4 (P2X4) (Tsuda et al., 2003) and type 7 (P2X7) (McGaraughty et al., 2007) in the development of nerve injury-induced neuropathic pain has been demonstrated in several animal models. In particular, the microglial P2X4 receptor expression is increased in parallel with the increase in pain hypersensitivity following nerve injury (Trang et al., 2012).

Acid sensing ion channels (ASICs) activated by extracellular acidosis also play an essential role in pain sensation (Gu and Lee, 2010). Spinal nerve root compression induces acid-sensing ion channel 3 (ASIC3) expression upregulation (Gu and Lee, 2010). In addition, the blockade of the acid-sensing ion channel 1a (ASIC1a) reduced

hyperalgesia in mice with a chronic constriction injury, and this reduction was mediated by the endogenous enkephalin pathway (Mazzuca et al., 2007).

Voltage-gated sodium (Na_V) channels are critical in the electrical excitability of sensory neurons and play a key role in pain sensation by controlling afferent impulse discharge. There are nine different isoforms ($Na_V1.1$-1.9). The $Na_V1.7$-1.9 are expressed mainly in the PNS and play a key role in the development and maintenance of chronic pain (Luiz and Wood, 2016). The $Na_V1.8$ channel is responsible for spontaneous action potential activity in damaged sensory axons, and contributes to the development of ectopic mechanosensitivity and neuropathic pain (Roza et al., 2003; Joshi et al., 2006). The subtype $Na_v1.3$ is also of special interest because it is not normally expressed in the PNS in adults; however this subtype suffers a dramatic upregulation in the DRG after peripheral nerve injury that contribute to the hyperexcitability and ectopic firing observed in spinal sensory neurons after injury (Lindia et al., 2005a).

In addition, decreases in the protein expression of voltage-gated K^+ (Kv) channels in sensory neurons have been shown in multiple rodent neuropathic pain models, which lead to a decrease in K^+ currents and in consequence to the hyperexcitation of sensory nerves (Tsantoulas and McMahon, 2014; Busserolles et al., 2016).

Finally, calcium channels are critical for pain neurotransmission and the activity of the N-type and T-type subtypes is increased in afferent fibers after different types of nerve injury (McGivern, 2006; Wen et al., 2010; Yue et al., 2013). The increment of activity of these channels decreases neuronal firing threshold, increases spontaneous firing, contribute to neurotransmitter release (such as glutamate) at primary afferent synapses and enhances firing evoked by repetitive stimulation (Saab, 2012).

1.2. Nerve injury produces changes in different anatomical locations

Peripheral nerve injury provokes a reaction in neurons, immune cells and glia at several anatomical locations: in the site of the lesion, in the dorsal root ganglia and in the central nervous system (Figure B). In fact, neuropathic pain is driven by neural plasticity, which can occur as both peripheral sensitization and central sensitization,

and leads to the development and maintenance of neuropathic pain. In this section, we describe the most important changes produced in these three locations of the nervous system, as a consequence of a lesion to a peripheral nerve, that contributes to neuropathic pain (Figure B).

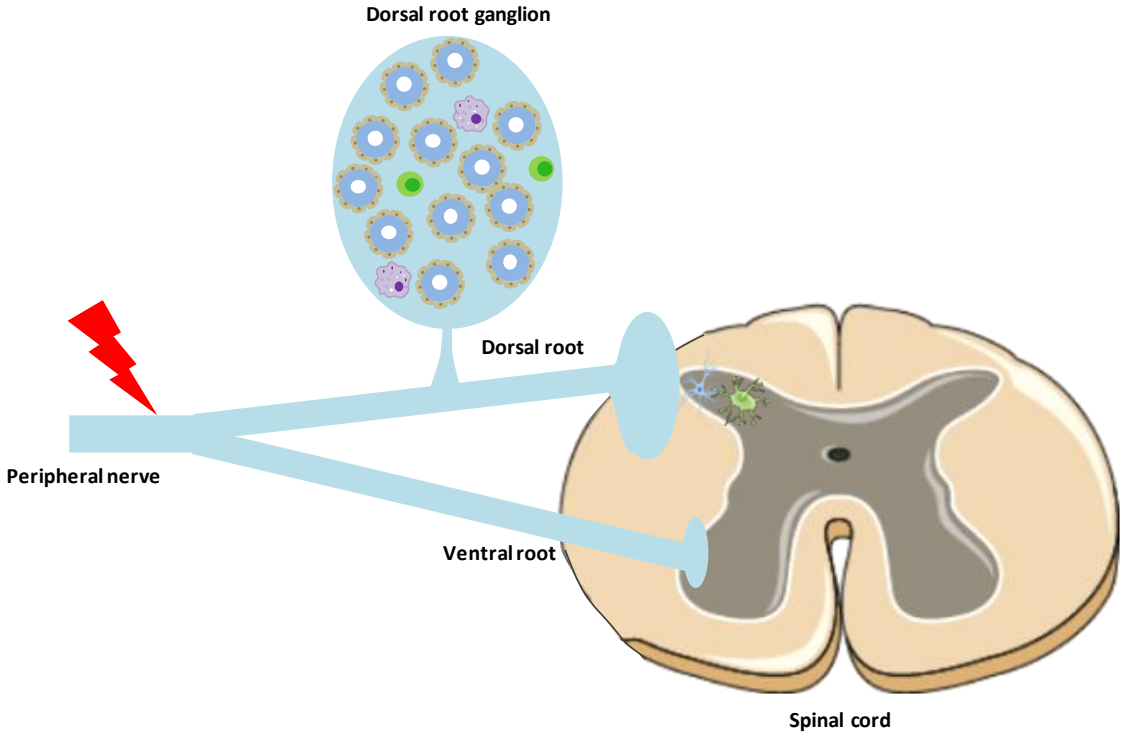

Figure B. Nerve injury provokes changes in the site of lesion, in the DRG, and in the ventral and dorsal horns of the spinal cord.

1.2.1. Changes in the site of the nerve lesion

Within hours after peripheral nerve injury, inflammatory mediators such as proinflammatory cytokines, chemokines, histamine, serotonin, bradykinin and prostaglandins are released from injured neurons and adjacent immune cells (resident mast cells, neutrophils, monocytes, macrophages and lymphocytes) (reviewed in Gao and Ji, 2010). The pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and the interleukins IL-1β, IL-6 and IL-18 also participate in the development of neuropathic pain (reviewed in Miller et al., 2009; Gao and Ji, 2010). These inflammatory mediators contribute to the recruitment of neutrophils and monocytes/macrophages and to the initiation of an inflammatory reaction that can contribute to the generation of pain (Zuo et al., 2003; Zhang and An, 2007). Anti-

inflammatory cytokines, such as the interleukins IL-4, IL-10, IL-11, IL-13, the leukemia inhibitory factor (LIF), the interferon-alpha (IFN-α) and the transforming growth factor beta (TGF-β), can also be released to control inflammatory response and attenuate pain (Zhu et al., 2001; Zhang and An, 2007; Liu et al., 2016b; Berta et el., 2017a).

After peripheral nerve injury, macrophages predominate in the initial inflammatory reaction but the first granulocytes that participate are neutrophils (reviewed in Scholz and Woolf, 2007). Neutrophils are attracted to the site of injury by the local release of leukotriene-B4, chemokine ligand 1 (CXCL1) and nerve growth factor (NGF) (Scholz and Woolf, 2007). Schwann cells attract macrophages to injury site by secreting chemokine (C-C motif) ligand 2 (CCL2, also named monocyte chemoattractant protein 1 [MCP-1]) and leukemia inhibitory factor (LIF) (Tofaris et al., 2002). Although neutrophil infiltration is relatively short-lived and limited to the lesion site, neutrophils release cytokines and chemoattractants that recruit macrophages during the first 24 h after injury (Perkins and Tracey, 2000). The upregulation of CCL2 is identified immediately after nerve injury (Taskinen and Röyttä, 2000) and is key for the recruitment of macrophages (Zhu et al., 2014; Zigmond and Echevarria, 2019). Macrophages are transformed into active phagocytes after nerve injury and this is shown by the upregulation of lysosomal markers and the inclusion of lipid droplets (Scholz and Woolf, 2007). Chemokine ligands (CCLs) 2 and 3, acting on the chemokine receptor 2 (CCR2) and the chemokine receptor 1 (CCR1), respectively, activate resident macrophages and induce monocytes to leave the bloodstream and enter the surrounding tissue to become tissue macrophages (Kwon et al., 2015a).

The recruitment of leukocytes from the circulation into injured nerve involves rolling along the endothelium (mediated by selectins), adhesion to the surface of the vascular endothelium (mediated by integrins), leukocytes extravasation and diapedesis, so that, leukocytes pass through the endothelium (Langer and Chavakis, 2009). Denervated Schwann cells (Schwann cells that do not contact with axons in the distal nerve stump after peripheral nerve injury) and activated macrophages secrete matrix metalloproteases that attack the basal lamina of blood vessels, leading to an interruption of the blood-nerve barrier in the entire distal nerve stump (Shubayev et al., 2006) and its restoration is followed by axonal regeneration in a proximal-distal

direction (Liu et al., 2008). Vasoactive mediators including substance P, bradykinin, calcitonin gene-related peptide (CGRP) and nitric oxide are released from injured axons to cause swelling and hyperemia (Zochodne et al., 1999). These vascular changes increase vascular permeability to promote the recruitment of immune cells into injured nerve, so approximately four days after injury a dense cellular infiltrate, mainly composed of T lymphocytes, macrophages, and mast cells, forms at the lesion site (Scholz and Woolf, 2007; Nadeau et al., 2011).

The number of macrophages and their phagocytic activity peak 7 days after a nerve transection and are maintained during several weeks in the injured nerve (Kwon et al., 2013). However, different macrophage time-courses in others neuropathic pain models have been described (Mueller et al., 2001; Nadeau et al., 2011).

In addition to pro-nociceptive mediators, immune cells (granulocytes, monocytes/macrophages and T lymphocytes) can also produce analgesic mediators, such as anti-inflammatory cytokines and opioid peptides, which counteract pain (Machelska, 2011; Plein and Rittner, 2017). In particular, it is known that opioid peptides released by immune cells can interact with opioid receptors that are synthesized in the DRG and are transported to the site of nerve injury to attenuate neuropathic pain (Machelska, 2011).

A peripheral nerve injury leads to the fragmentation and degeneration of the distal nerve stump referred to as Wallerian degeneration, which is an active process of self-destruction of the nerve that can work independently of non-neuronal cells surrounding the axon (Saxena and Caroni, 2007). Wallerian degeneration consist of cellular and molecular alterations, including macrophage invasion, activation of Schwann cells, as well as release of chemical signals, such as neurotrophins, and cytokine upregulation (Dubový, 2011). These chemical signals include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and glial cell line-derived neurotrophic factor (GDNF), which are retrogradely transported to the cell bodies of primary sensory neurons where they act as potent regulators of gene expression and support the nervous system's growth, maintenance, and survival (Sah et al., 2006; Khan and Smith, 2015). NGF and GDNF also directly activate and

sensitize nociceptors (Malin et al., 2006), contributing to the initiation of pain in response to nerve injury. Upregulation of cytokines, such as TNF-α and IL-1β, also play a crucial role in the initiation of these degenerative processes (Shamash et al., 2002; Perrin et al., 2005).

Removal of distal degenerating axons and myelin debris by phagocytosis enables a reorganization of Schwann cells and lays the foundation for the regrowth and remyelination of injured axons (Stoll et al., 2002). Impaired axonal transport probably triggers axonal degeneration (Mack et al., 2001). Schwann cells carry out the first step in the myelin sheath evacuation by myelin fragmentation. Then, the degraded myelin is phagocytized by the recruited macrophages to obtain full myelin clearance (Dubový, 2011). It is important that Schwann cells and macrophages cooperatively achieve myelin phagocytosis, although the molecular mechanisms of myelin phagocytosis by Schwann cells and macrophages are thought to be different. In response to IL-1β, macrophages also participate in the production of mitogenic factors for Schwann cells and fibroblasts and induce the synthesis of NGF (Dubový, 2011). There are evidences that CCL2 and IL-1β are important mediators for the modulation of macrophage responses that lead to fast myelin disruption and debris clearance in Wallerian degeneration (Perrin et al., 2005). Macrophages in the injured nerve are eliminated by local apoptosis and circulation into regional lymph nodes when ends the Wallerian degeneration (Hu and McLachlan, 2003; Kuhlmann et al., 2001) (Figure C)**.**

Last but not least, keratinocytes can activate nociceptors since they reside near the peripheral terminals of nociceptors and produce several neuroactive mediators such as IL-1β, ATP, NGF, endothelin, and prostaglandin E2 that are proalgesics (Ji et al., 2016).

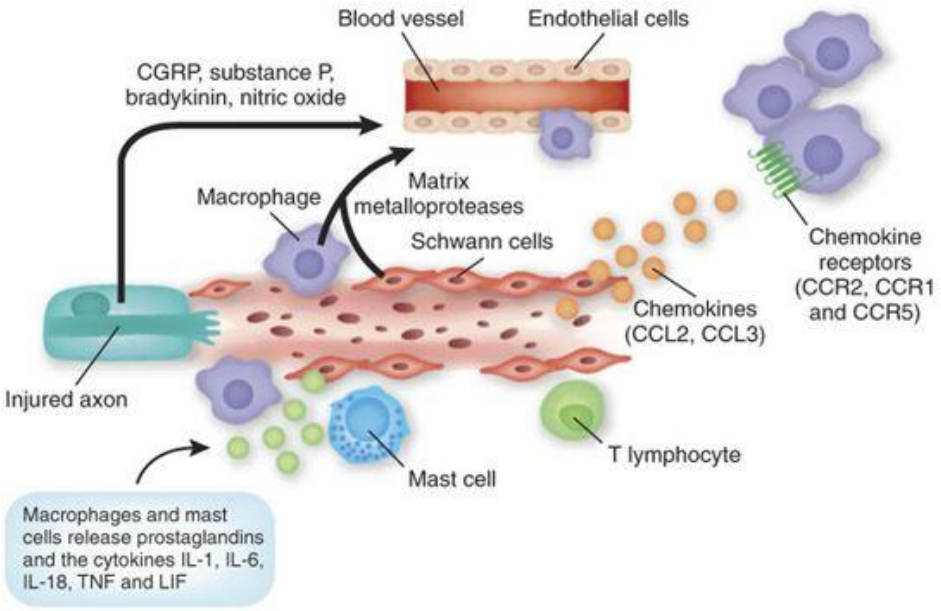

Figure C. Inflammatory changes associated with wallerian degeneration. Schwann cells and macrophages produce matrix metalloproteases that interrupt the blood-nerve barrier. Substance P, bradykinin, calcitonin gene-related peptide (CGRP) and nitric oxide released by injured nerve fibers induce hyperemia and swelling, encouraging the infiltration of monocytes and T lymphocytes. Monocytes are attracted and guided by the cytokine TNF-α and the chemokines CCL2 and CCL3 to the lesion site. Macrophages and mast cells release prostaglandins and the cytokines TNF-α, LIF, IL-1β, IL-6 and IL-18. Macrophage infiltration is also promoted by TNF-α *(Figure taken from Scholz and Woolf, 2007).*

1.2.2. Changes in the DRG after nerve injury

1.2.2.1. The cell body response to nerve injury

Separation of an axon from its cell body produces several morphological changes in the perikarya (soma of neuron). Chromatolysis is a reactive response that occurs in the cell body of damaged neurons, involving the disruption, dispersal and redistribution of Nissl substance (rough endoplasmic reticulum) leaving clear areas of empty cytoplasm (Sterman and Delannoy, 1985) (Figure D). During this process, the neuronal nucleus is displaced of its usual central location to the cell periphery, showing an eccentric position (Johnson and Sears, 2013) (Figure D). It has been proposed that these changes in the Nissl substance and in the nucleus may reflect a change from the synthesis of proteins involved in synaptic transmission (neurotransmitters or neuromodulators) to the synthesis of proteins involved in nerve regeneration (Matthews and Raisman, 1972).

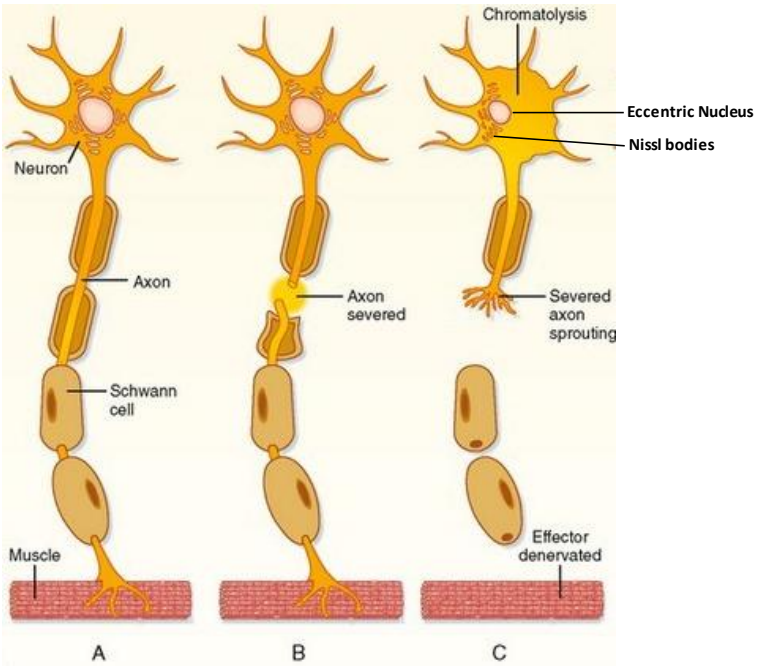

Figure D. Scheme of a neuron undergoing chromatolysis. After a healthy neuron (A) suffers an injury to the axon (B), Nissl bodies migrate together with the nucleus towards the periphery of the cell (C). *(Figure taken from Berne et al., 2009 with slight modification).*

These events could be triggered by changes in the expression of specific transcription factors including, cJUN, STAT3, and ATF3 (Chandran et al., 2016). ATF3 (Activating Transcription Factor 3) is a reliable marker of cellular stress (Tsujino et al., 2000). ATF3 is not expressed in neurons in normal conditions, but following the cut of a peripheral nerve, ATF3 is immediately induced in virtually all DRG neurons and motoneurons that are axotomized, and the time course of induction is dependent on the distance between the injury site and the cell body. Therefore, ATF3 is considered as a neuronal marker of nerve injury (Tsujino et al., 2000) (Figure E). A neuronal nuclei antigen called NeuN is a well-recognized neuronal marker that is distributed in the nuclei of mature neurons in nearly all parts of the vertebrate nervous system. However, Collombet and colleagues found that after brain lesions NeuN antigenicity is reduced in the damaged neurons (Collombet et al. 2006).

1.2.2.2. Gene expression after peripheral nerve injury

After nerve injury there are changes in the expression of genes encoding for neuropeptides, receptors, ion channels, signal transduction molecules, synaptic vesicle proteins, and other proteins in DRG neurons, which suggest that there are dynamic and complex changes in the molecular diversity in DRG neurons (Xiao et al., 2002; Cobos et al., 2018). In addition, a marked upregulation of genes in the DRG that are related to immune cell function reflects the extent of both the recruitment and activity of macrophages and T cells and underscores the substantial changes that occur in the local environment of primary sensory neurons after axonal injury (Costigan et al., 2002).

It has been described that large sensory neurons expressed transcripts associated with glucose, survival and proliferation functions while small DRG neurons expressed transcripts associated with oxidative stress (e.g. increase of caspase-6) after SNI and paclitaxel-induced neuropathy. These transcriptional changes are found mainly in the injured DRG neurons (Berta et al., 2017b). The studies of changes in global transcript expression could help to understand the pathophysiological mechanisms that occur in a neuropathic process and the mechanistic differences between the different pain modalities. A study that correlates the time-course of changes in expression array with the changes in pain hypersensitivity to different kind of stimuli showed that cold allodynia correlates with transcripts related with the nociceptors, whereas tactile hypersensitivity correlates with immune cell transcripts (Cobos et al., 2018).

1.2.2.3. Neuro-Immune Interactions

Although resident immune and glial cells in the DRG are away from the primary lesion site, these cells react to peripheral nerve injury, and their response is reinforced by invading macrophages and T cells (Scholz and Woolf, 2007) (Figure E). The activation of these cells also leads to the production of pro-nociceptive mediators that sensitize and reduce the threshold of neuronal firing, leading to peripheral sensitization that can induce subsequent central sensitization, and both contribute to neuropathic pain (Okamoto et al., 2001; DeLeo et al., 2004; Gao and Ji, 2010) (Figure E). The pro-

inflammatory mediators produced by neurons, satellite glial cells, macrophages and lymphocytes within the DRG include cytokines, such as interleukins (IL-1β and IL-6) (Sweitzer et al., 2001; Zalenka et al., 2005), TNF-α (Zalenka et al., 2005; Ogawa et al., 2014), chemokines (White et al., 2007; Gao and Ji, 2010), and interferons (Chauhan et al., 2018), but also eicosanoids (Martin et al., 2019), growth factors (Tender et al., 2013; Terada et al., 2018), ATP (Chen et al, 2016) and reactive oxygen species (Branca et al., 2018).

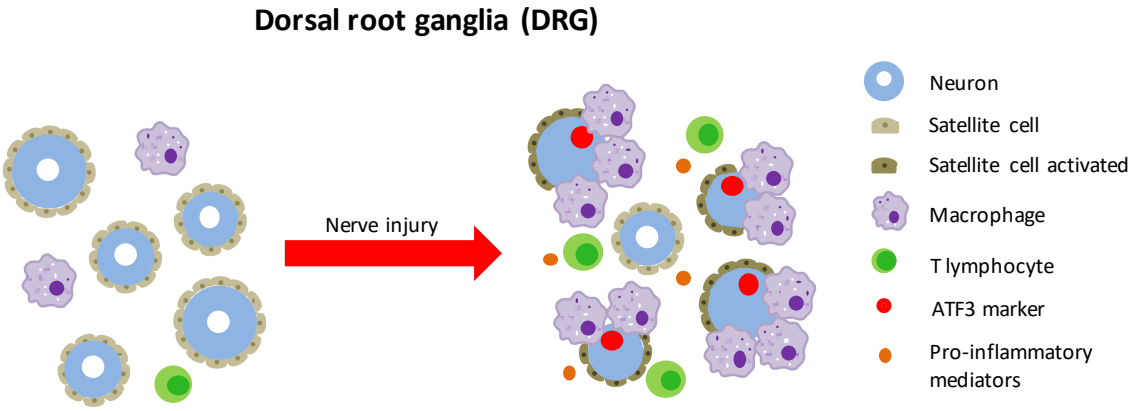

Figure E. Changes in neurons and immune cells induced by nerve lesion. Injured neurons express ATF3. Macrophages and a few T lymphocytes reside in the DRG before injury. Their numbers pronouncedly increase after injury. Macrophages also move around the cell bodies of injured primary sensory neurons. Satellite cells that surround lesioned neurons increase the expression of glial fibrillary acidic protein and are activated. Neurons, satellite cells, macrophages and lymphocytes release pro-inflammatory mediators.

Neutrophils are only found in the DRG, in significant numbers, in experimental nerve lesions that involve inflammation, such as the chronic constriction injury model (Scholz and Woolf, 2007). However, these granulocytes are not relevant in the DRG after axotomy (Lindborg et al., 2018). Injury-induced macrophage invasion appears to be triggered by the release of the chemokine fractalkine (also named CX3CL1) (Zhuang et al., 2007; Huang et al., 2014) and the chemokine CCL2 (Zhang et al., 2016; Zigmond and Echevarria, 2019) from DRG neurons (Figure F). Fractalkine and CCL2 activate the C-X3-C motif chemokine receptor 1 (CX3CR1) and CCR2 receptors, respectively, which are expressed by macrophages. However, there are controversial results regarding the fact that DRG neurons truly release fractalkine (Montague and Malcangio, 2017). Morphological and electrophysiological studies showed that CCR2 was upregulated in

injured DRG neurons and macrophages after nerve injury (White et al., 2005a; Zhu et al., 2014). CCL2 increments the sensitization of primary afferent neurons (Abbadie et al., 2003) and recruits macrophages (Zhu et al., 2014) when binds to CCR2 in neurons and macrophages, respectively. (Figure F). Therefore, CCL2 and CCR2 upregulation induced by nerve injury may contribute to the pain behavior through the regulation of both neuronal excitability and macrophage infiltration in the DRG (Zhu et al., 2014) (Figure F). In fact, CCR2 knockout mice and CCL2 neutralizing antibodies in wild-type (WT) mice prevent infiltration of macrophages and mechanical hypersensitivity produced during neuropathic pain (Abbadie et al., 2003; Zhang et al., 2016).

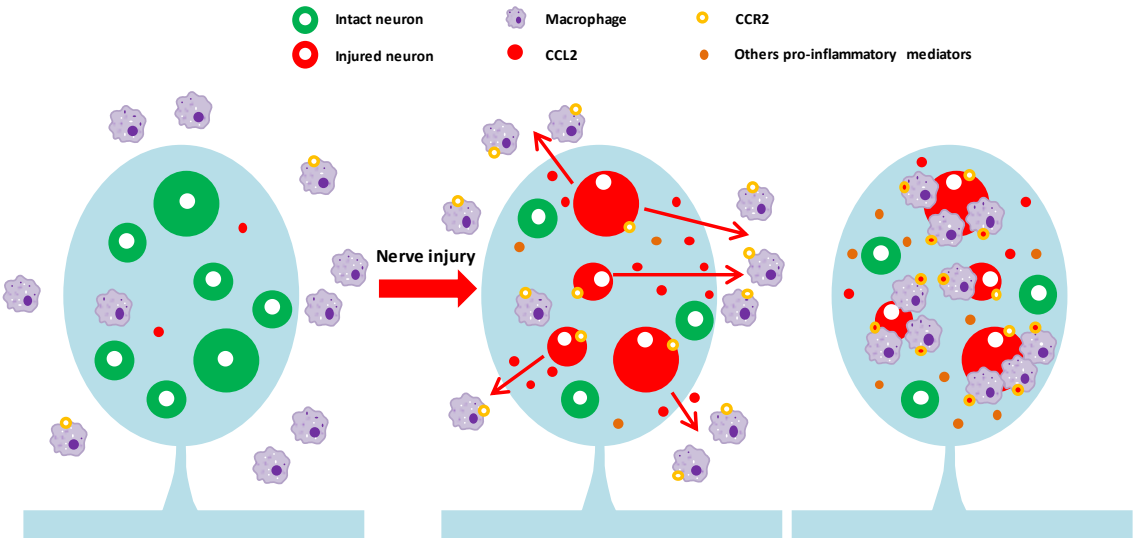

Figure F. Diagram of how macrophages, CCL2 and CCR2 are regulated in the DRG after nerve injury. In the DRG, nerve transection induces an increase of CCL2 in neurons and an increment of chemokine receptor CCR2 in neurons and macrophages. CCL2 may act on CCR2 in neurons to increase neuronal excitability and in macrophages to induce their infiltration into the DRG. Pro-inflammatory mediators are released by neurons and macrophages that sensitize the intact neurons.

The time course of macrophage and T lymphocyte infiltration in DRG differs depending on sex (Sorge et al., 2015), age (Costigan et al., 2009a), species and neuropathic pain model used (Hu and McLachlan, 2002; Ristoiu, 2013) in each study. Seven days after nerve transection, macrophages move from an initially rather diffuse distribution in the DRG to surround the cell bodies of injured sensory neurons (Figures E and F) (Vega-Avelaira et al., 2009). Two months after a peripheral nerve transection, a substantial proportion of macrophages in the DRG turn into active phagocytes, presumably

removing debris from injured sensory neurons (Hu and McLachlan, 2003), which begin to degenerate after axotomy.

Macrophages have several phenotypes associated to their function, there are pro-inflammatory macrophages (M1) and anti-inflammatory macrophages (M2), which play distinct roles in the induction and resolution of pain (Ji et al., 2016). M2 macrophages release higher amounts of opioid peptides, including Met-enkephalin, dynorphin A, and β-endorphin in comparison to M1 (Pannell et al., 2016). These opioid peptides attenuate the excitability of neurons and the release of proinflammatory and proalgesic neuropeptides, such as substance P or CGRP, from peripheral terminals of nociceptors, reducing the pain hypersensitivity (Machelska, 2011). Furthermore, the administration of minocycline (a microglia/macrophage inhibitor) reduces the upregulation of the precursors of dynorphin and β-endorphin in the DRG after chronic constriction injury (CCI), suggesting that opioid peptides are released by the macrophages in the DRG after CCI to attenuate the neuropathic pain (Mika et al., 2010).

In the rat, a decrease in small unmyelinated neurons, is not detectable 4 weeks after nerve transection (Tandrup et al., 2000), while in the mouse, after a week, their number is decreased dramatically (Shi et al., 2001). The ongoing loss of sensory neurons may therefore, be one factor responsible for the sustained increase in the number of macrophages and T lymphocytes in the DRG.

Several months after nerve injury, macrophages and T cells are also closely associated with sympathetic nerve fiber terminals that sprout into basket-like structures around large-diameter sensory neurons (Scholz and Woolf, 2007). Sprouting of noradrenergic terminals is reduced in mice lacking IL-6, indicating that this cytokine is one of the signals that trigger the sympathetic nerve fiber invasion (Ramer et al., 1998). The formation of sympathetic fiber baskets furthermore depends on satellite cell-derived NGF and neurotrophin-3, suggesting coordinated communication between macrophages and satellite cells (Figure G). In addition, satellite cells may be the origin of neural crest progenitors for neurons and Schwann cells that could help repopulate the DRG after injury (Li et al., 2007).

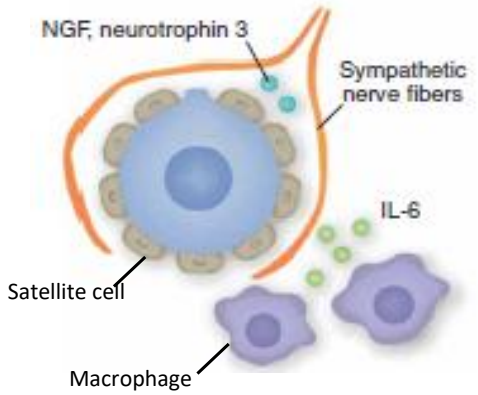

Figure G. Involvement of immune response in sympathetic nerve sprouting into the DRG. IL-6, which is released from macrophages, triggers the sprouting of sympathetic nerve fibers into the DRG. Noradrenergic fiber baskets formed around large-diameter sensory neurons also depend on NGF and neurotrophin-3, which are released from satellite cells *(Figure taken from Scholz and Woolf, 2007 with slight modifications).*

1.2.3. Changes in the spinal cord after nerve injury

When a peripheral nerve is injured, astrocytes and microglia are activated by cytokines and other mediators, such as ATP, secreted from the central terminals of injured peripheral nerves in the spinal cord. Upon activation, astrocytes and microglia can release large amounts of proalgesic agents in the spinal cord (Miller et al., 2009). These proalgesic agents are among others neuropeptides (CGRP and substance P), neurotransmitters (glutamate), neurotrophic factors (NGF, BDNF), chemokines (CCL2) and cytokines (TNF-α, IL-1β, IL-6) (Obata and Noguchi, 2008). These mediators produce neuroexcitation and/or disinhibition on primary afferent neurons, pain projection neurons and interneurons, which can trigger painful sensitization (Li and Grace, 2019).

Thus, similarly to what happens in the peripheral nervous system, peripheral nerve injury leads to a neuroinflammatory process in the central nervous system characterized by an increased activation of astrocytes and microglia. Compared with the microglial response, astrocyte activation in the side of the spinal cord ipsilateral to the injury, begins relatively late and progresses slowly, but is sustained for a longer period (more than 5 months) (Zhuang et al., 2005; Zhang and De Koninck, 2006; Echeverry et al., 2008; Thakur et al., 2017). Therefore, activation of microglia may have more relevance for the generation of neuropathic pain while activation of astrocytes

may be related to the maintenance of neuropathic pain (Yan et al., 2017). Like macrophages, microglia cells have diverse functional states that range from the pro-inflammatory "M1" phenotype to the anti-inflammatory and pro-resolute "M2 phenotype" (Ji et al., 2018). In line with this “M1/M2” terminology for macrophages and microglial cells, it has been proposed the nomenclature “A1” and “A2” for astrocytes (Liddelow and Barres, 2017). A1 phenotype has "harmful" functions, while A2 phenotype acts as anti-inflammatory, promoting the survival and growth of neurons (Liddelow and Barres, 2017; Ji et al., 2018).

After a nerve lesion, microglial cells form dense clusters around the cell bodies of injured motor neurons in the ventral horn and in the vicinity of the central terminals of injured sensory nerve in the dorsal horn of the spinal cord (Watkins and Maier, 2002; Beggs and Salter, 2007). Spinal microglial activation in both dorsal and ventral horns peaks a week after injury, followed by a slow decline over several weeks. This temporal pattern differs from that of the very early inflammatory reaction distal to a nerve lesion site and the sustained infiltration of macrophages and lymphocytes in the DRG, suggesting that the central immune response to peripheral nerve injury is independently organized and has distinct functional consequences (Scholz and Woolf, 2007).

Colony-stimulating factor 1 (CSF1) is a cytokine involved in the proliferation and differentiation of microglia and macrophages, which has been reported to serve as a new messenger for chronic pain, and could be a trigger for microglial activation (Thuault, 2016). Additional signaling pathways mediate the activation of resident spinal microglia and probably also the recruitment of circulating monocytes to the dorsal horn. These involve the chemokine fractalkine acting on the receptor CX3CR1 (Verge et al., 2004), CCL2 signaling through the receptor CCR2 (White et al., 2005b), and ATP through activation of P2X4 and P2X7 receptors and Toll-like receptors (Tanga et al., 2005; Kim et al., 2007) (Figure H).

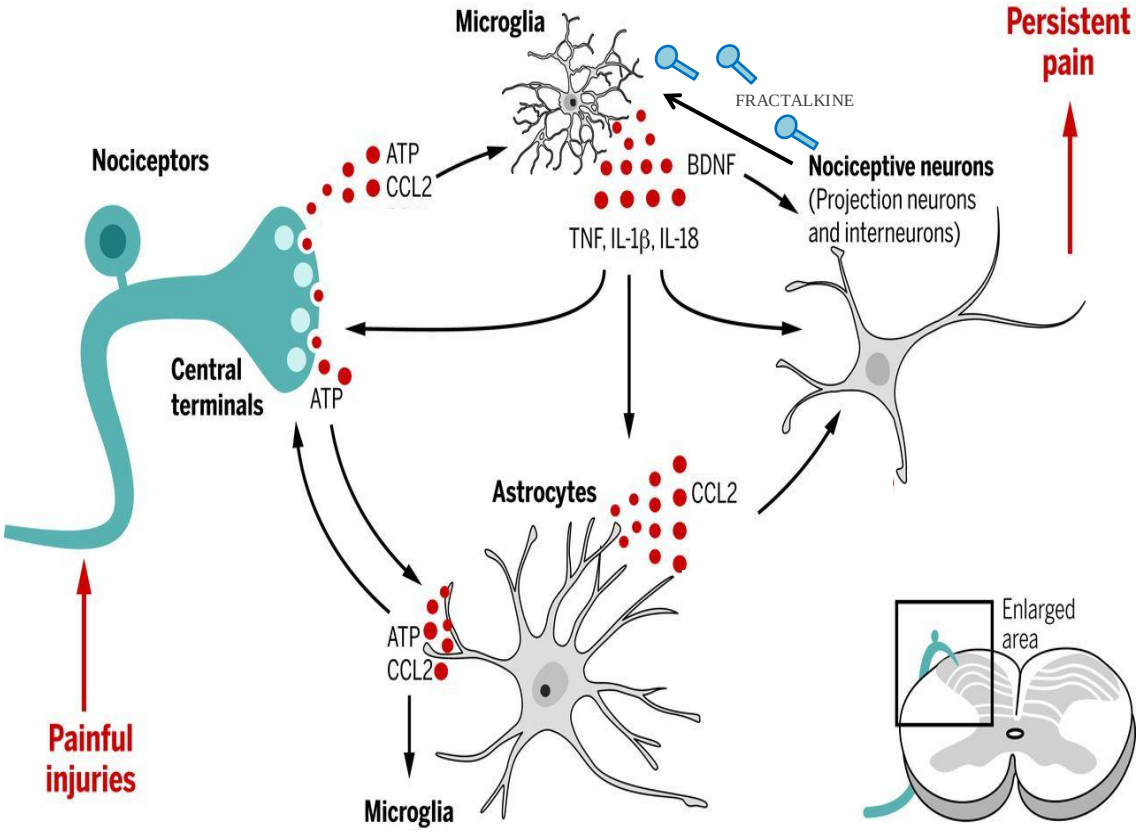

Figure H. Neuroimmune interactions in the spinal cord involved in neuropathic pain. Nerve injury produces hyperactivity of nociceptors and release of modulators from their central terminals, so that microglia and astrocytes are activated of in the dorsal horn of the spinal cord. Activated microglia and astrocytes secrete neuromodulators that produce neuronal and synaptic plasticity. There is a communication between the pre- and postsynaptic neurons and microglia/astrocytes *(Figure taken from Ji et al., 2016 with slight modifications).*

Fractalkine (also named CX3CL1) is an atypical chemokine that is constitutively expressed by neurons (Lindia et al., 2005b). This chemokine exists in either a membrane-bound form and as a cleaved soluble chemokine but it is active in both forms (Imai et al., 1997). Fractalkine is also expressed in endothelial cells where it acts as an adhesion molecule on its membrane-bound form, allowing leucocyte adhesion (leucocytes such as monocytes, NK cells, and T cells expressed CX3CR1 that binds to membrane-bound fractalkine) (Imai et al., 1997). Under inflammatory conditions, the membrane-bound form can be cleaved to release a soluble chemokine that acts as a chemoattractant for monocytes and T cells (Imai et al., 1997). After nerve injury, CX3CR1 is upregulated in microglia that also release proteases such as cathepsin S, which cleaved the membrane-bound fractalkine in the spinal neurons allowing the release of the soluble fractalkine; this chemokine released from neurons in the spinal cord can further activate microglia (Lindia et al., 2005b; Clark and Malcangio, 2012). Fractalkine-mediated signaling between neurons and glia through CX3CR1 seems to

contribute to the development of neuropathic pain (Verge et al., 2004). Intrathecal injection of fractalkine produces mechanical allodynia and thermal hyperalgesia (Milligan et al., 2004; Zhuang et al., 2007), whereas administration of a neutralizing antibody against CX3CR1 delays the development of mechanical allodynia after chronic constriction of the sciatic nerve and spinal nerve ligation (Milligan et al., 2004; Zhuang et al., 2007).

CCL2 is expressed by primary sensory neurons in the DRG (White et al., 2005a, Zhang and De Koninck, 2006) and after peripheral nerve injury is released by the primary afferents in the dorsal horn of the spinal cord (Van Steenwinckel et al., 2011). Microglial cells express the receptor CCR2, suggesting a potential pathway of direct communication between injured primary afferent fiber terminals and dorsal horn microglia (Abbadie et al., 2003) (Figure H).

After peripheral nerve injury, the central terminals of sensory neurons can also release ATP which acts on spinal cord microglia, through activation of P2X4 and P2X7 receptor, and this leads to BDNF and IL-1β release, respectively (Costigan et al., 2009b) (Figure H). The release of BDNF by microglia depolarizes spinal lamina I projection neurons through the downregulation of the K^+-Cl^- cotransporter KCC2, which results in a dysregulation in the Cl^- gradient, such that activation of GABA-A receptors depolarizes, rather than hyperpolarizes (Costigan et al., 2009b). The blockade of BDNF reverses the allodynia after nerve injury, so that BDNF is consider a crucial signaling molecule between microglia and neurons (Figure H) (Coull et al., 2005). In addition, both IL-1β and TNF-α can downregulate transporters expressed by astrocytes that are responsible for the uptake of glutamate to maintain the homeostatic concentrations of extracellular glutamate and, in consequence, high aberrant concentrations of glutamate are kept in the synaptic cleft and this contribute to the neural overexcitation (Li and Grace, 2019).

Mammalian Toll-like receptors (TLRs) are a family of twelve evolutionarily conserved membrane proteins that are expressed on immune cells and glial cells, which function to protect the organism initiating inflammatory response to tissue damage or injury (Trinchieri and Sher, 2007, Lacagnina et al., 2018). TLRs are expressed both at

peripheral (Schwann cells, satellite glial cells, fibroblasts, endothelial cells, macrophages, and sensory neurons) and at central (microglia, astrocytes, oligodendrocytes) level in the nervous system (Lacagnina et al., 2018). TLRs recognize nucleic acids and proteins released after cell damage, so that they can be activated by cellular debris from the degenerating central terminals of injured afferents or in response to the nerve injury (Scholz et al., 2005). Furthermore, there is a link between neuropathic pain and the innate immune response mediated through TLR activation. In mice lacking toll-like receptor subtype 2 (TLR2) (Kim et al., 2007), toll-like receptor subtype 3 (TLR3) or toll-like receptor subtype 4 (TLR4) (Tanga et al., 2005), microglial activation and the induction of proinflammatory cytokines after a peripheral nerve lesion are substantially diminished. Moreover, these animals also show less neuropathic pain-like behavior (Tanga et al., 2005; Kim et al., 2007).

Microglial activation in the dorsal horn is also accompanied by an invasion of T lymphocytes (Hu et al., 2007). T-cell-deficient mice develop less neuropathic mechanical allodynia indicating that T cells contribute to the evolution of neuropathic pain (Costigan et al., 2009a; Cobos et al., 2018).

Similarly to non-neuronal cells in the peripheral nervous system, microglia, astrocytes and T cells can also release antinociceptive cytokines (e.g.: IL-10, IL-4) in the spinal cord (Ji et al., 2016). In addition, it has been found that microglia can produce opioid peptides such as β-endorphin and their release is blocked by the microglial inhibitor minocycline (Fan et al., 2016). However, little is known about under what circumstances these non-neuronal cells release anti-nociceptive mediators. Interestingly, an imbalance of pro- and anti-inflammatory cytokines has been described in cerebrospinal fluid of patients with chronic pain, which may contribute to the maintenance of pain (Backonja et al., 2008; Lundborg et al., 2010).

Finally, the activation of glial cells associated to chronic pain described in preclinical studies has also been demonstrated in clinical studies using brain imaging. Thus, Loggia and collagues showed an increment of glial activation in the brain of patients with chronic low back pain (Loggia et al., 2015) and fibromyalgia (Albrecht et al., 2018).

To sum up, several changes such as the activation of microglia and astrocytes or the release of pronociceptive cytokines occur in the spinal cord after nerve injury and these changes could participate in the central sensitization described in the next section.

1.3. Central sensitization

IASP defines central sensitization as "increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input". Central sensitization can be triggered by an intense and sustained nociceptive stimulation, after noxious inflammation or after peripheral or central nervous system damage, which induces a sustained release of glutamate and several neuropeptides from the central terminals of primary afferent neurons (reviewed by Latremoliere and Woolf, 2009). In fact, several mechanisms contribute to central sensitization, such as glutamatergic neurotransmission (acting on N-methyl-D-aspartate [NMDA], α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [AMPA], and metabotropic glutamate [mGlu] receptors) and the release from central terminals of primary afferent neurons of substance P, CGRP, bradykinin and BDNF (which activate neurokinin 1 [NK1] receptors, CGRP receptors, bradykinin 2 [B2] receptors and tropomyosin receptor kinase B [TrkB] receptors, respectively) (Figure I). Among these mechanisms, the activation of NMDA receptors, which under normal conditions are silent, by a sustained release from nociceptors of glutamate is a key step for both the initiation and maintenance of central sensitization. Activation of NMDA receptors in spinal projection neurons, allows the entry of Ca^{2+} into these neurons and therefore contributes to their depolarization (Latremoliere and Woolf, 2009).

There are many parallel intracellular inputs to dorsal horn neurons that can cooperatively or/and independently initiate central sensitization but the elevation of intracellular calcium is the major of them (Latremoliere and Woolf, 2009). A relevant role is also played by the extracellular signal-regulated kinases (ERK), which are triggered by multiples inputs, such as the activation of protein kinase A (PKA), calcium/calmodulin-dependent protein kinase II (CaM-KII) and protein kinase C (PKC).

The phosphorylation of ERK increases the AMPA and NMDA currents and reduces potassium currents (Figure I), and for that reason ERK phosphorylation in neurons is considered a standard marker for central sensitization (Gao and Ji, 2009). Other intracellular mediators of central sensitization are PKA and PKC, which phosphorylate the NR1 subunit of the NMDA receptor to increase its response to glutamate, increasing the pain hypersensitivity (Latremoliere and Woolf, 2009). In particular, PKC activation contributes to hyperexcitability of spinal cord nociceptive neurons by two different pathways. The first one is by reducing the silent state of NMDA receptors produced by magnesium ion sitting in the receptor pore, increasing the probability of channel opening. The second one is by reducing the tonic inhibition by gamma-aminobutyric acid (GABA) and glycine of spinal cord neurons. This disinhibition of the dorsal horn neurons produces greater susceptibility to activation by excitatory inputs (Latremoliere and Woolf, 2009).

In summary, multiple processes contribute to central sensitization such as: increased membrane excitability, facilitated synaptic strength and decreased inhibitory influences in dorsal horn neurons. These processes are consequence of changes in the activation of NMDA and AMPA receptors and in their trafficking to the membrane, alterations in ion channels and reductions in the release or activity of GABA and glycine (Latremoliere and Woolf, 2009). In addition to changes in pain transmission neurons, inhibitory interneurons and descending modulatory control systems are dysfunctional in patients with neuropathic pain (Colloca et al., 2017). Consequently, the brain receives altered and abnormal sensory messages (Colloca et al., 2017).

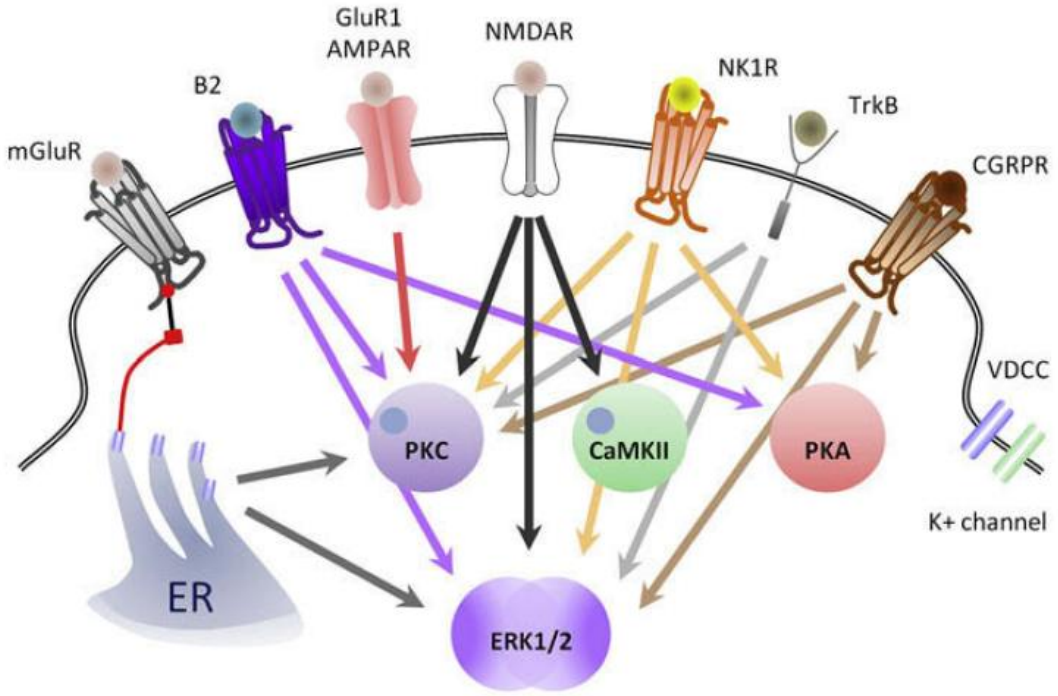

Figure I. Contribution of glutamate (acting on mGluR, AMPAR and NMDAR receptors), bradykinin (on B2 receptor), substance P (on NK1R receptor), BDNF (on TrkB receptor) and CGRP (on CGRPR receptor) to central sensitization. Activation of protein kinase A (PKA), calcium/calmodulin-dependent protein kinase II (CaM-KII) and protein kinase C (PKC) produces the phosphorylation of ERK that activates NMDA and AMPA receptors. ERK also produces a decrease in K^+ currents, increasing membrane excitability *(Figure taken from Latremoliere and Woolf, 2009).*

The release of cytokines, including IL-1β (Stemkowski et al., 2015), IL-6 (Kawasaki et al., 2008; Dubový et al., 2010) and TNF-α, (Leung and Cahill, 2010) by activated astrocytes and microglia can directly modulate neuronal activity and elicit spontaneous action potential discharges, increasing the release of glutamate from primary afferents (Yan and Weng, 2013), and decreasing the release of GABA from dorsal horn interneurons (Wieseler-Frank et al., 2005; Zhang and Dougherty, 2011). In fact, the inhibition of the IL-1β signaling leaded to the attenuation of spontaneous sensory neuron firing (Wolf et al., 2006), and neuropathic pain-like behavior (Wolf et al., 2006).

In the absence of injury or infection, noxious mechanical, chemical or thermal stimuli are detected by nociceptors (Aδ or C fibers) (Yan et al., 2017), whose afferents end in the lamina I and II of the spinal cord dorsal horn, whereas low-threshold mechanoreceptive afferents (Aβ fibers) do not produce excitability of pain sensory neurons. However, after peripheral nerve injury, nociceptors are highly activated and Aβ fibers arborize into laminal I or II, establishing new synaptic connection with the

existing nociceptive neurons. This phenomenon is called “sprouting” (Yan et al., 2017) and these structural changes also contribute to altered synaptic function. Under this condition, Aβ fibers increase sensitivity to pain producing hyperalgesia.

In many models of neuropathic pain, a decrease in the release or activity of inhibitory neurotransmitters leads to a state of disinhibition (Latremoliere and Woolf, 2009). In particular, there is substantial disinhibition in the dorsal horn probably as a consequence of apoptosis of inhibitory interneurons due to glutame excitotoxicity that lead to a reduction of GABAergic and glycinergic inhibitory currents (Latremoliere and Woolf, 2009). Furthermore, there is evidence that the noradrenergic inhibitory control fails (Bannister and Dickenson, 2016). In normal animals, noradrenaline activates the α_2-adrenoceptor in the spinal cord producing an antinociceptive effect (Bannister and Dickenson, 2016). However, a peripheral nerve injury produces a suppression of noradrenergic spinal α_2-adrenoceptor-mediated inhibition (Rahman et al., 2008). On the other hand, in neuropathic pain there is an increment in the descending facilitatory control through the activation of serotonin subtype 3 (5-HT_3) receptor (Suzuki et al., 2004).

In conclusion, multiple sites from skin to brain level are altered after nerve injury and several mechanisms are involved in the development of the neuropathic pain. Many of them have been explained in this chapter and summarized in the figure J.

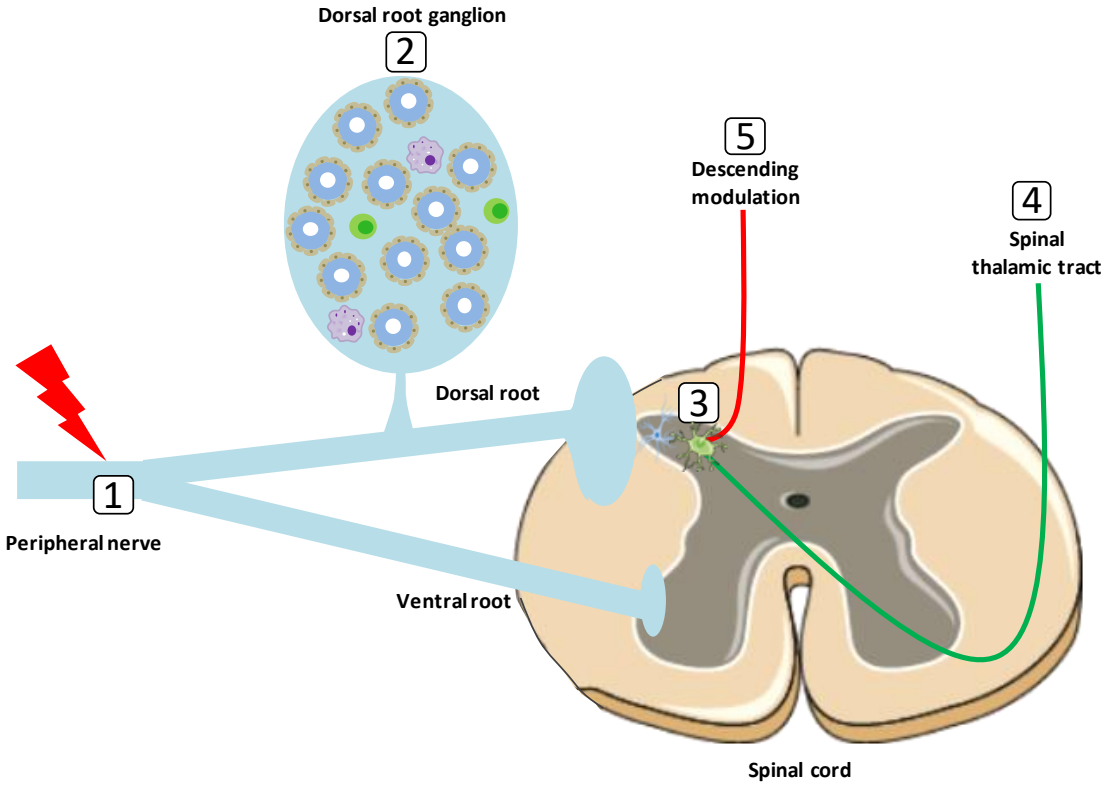

Figure J. Nerve injury produces changes in multiple sites along the somatosensory nervous system. (1) Spontaneous ectopic activity develops at the site of nerve injury. Partial denervation triggers infiltration of immune cells (neutrophils, macrophages, T cells...) at the site of nerve injury that release pro-inflammatory mediators that sensitizes primary afferent nociceptors. The distal part of the injured nerve is degenerated during the process of Wallerian degeneration. (2) Up- or down-regulation of different pain mediators are also produced in the dorsal root ganglia following nerve injury. Spontaneous ectopic neural activity develops in the dorsal root ganglia. Injured neurons express ATF3. Infiltration of immune cells (macrophages, T cells) also takes place. (3) Sensitization of the postsynaptic dorsal horn neurons occurs as a consequence of the release of pronociceptive mediators both by primary afferent neuron terminals and by microglia and astrocytes. Sprouting of Aβ fibers to lamina I and II also happens after long-duration injury. (4) Sensitization of ascending pathways to thalamus. (5) Inhibition of descending inhibitory pathways.

2. SIGMA-1 RECEPTORS AND PAIN

2.1. The discovery of sigma receptors

The Sig-1R were firstly misclassified by Martin and coworkers in 1976 as a subclass of opioid receptors (Martin et al., 1976). In 1982, Su and colleagues demonstrated the existence of a "sigma receptor" that differed from other known opioid receptors (Su, 1982). Later, sigma receptors were characterized into two subtypes, based on their pharmacological profile, which were termed as sigma-1 and sigma-2 receptors (Quirion et al., 1992). The Sig-1R was cloned for the first time in guinea pig (Hanner et al., 1996), and later in other species such as rats, mice, and humans (Pan et al., 1998).

The Sig-1R is an unusual transmembrane protein implicated in different cellular functions and with possible roles in both normal and disease states in humans (Hayashi and Su, 2007). Since its discovery, the Sig-1R has been implicated in diverse pathophysiological conditions ranging from cancer (Rizzuto et al., 2004) to neurodegenerative diseases (Maurice and Su, 2009) and chronic pain (Sánchez-Fernández et al., 2017).

Although the Sig-1R was cloned 20 years ago, its structure is still controversial. It is know that this receptor is a 29-kDa single polypeptide composed by 223 amino acids, and without homology with opioid receptors or with any other known mammalian protein (Cobos et al., 2008; Su et al., 2010). However, there are controversies about its tridimensional structure, it was proposed that it has two transmembrane domains, but when Sig-1R was cristalized a model with three transmembrane domains has been proposed (Chu and Ruoho, 2016; Schmidt et al., 2016). The gen for the human Sig-1R is located in the cromosome 9 band p13 and contains three introns and four exons (Chu and Ruoho, 2016). The development of Sig-1R knockout (Sig-1R-KO) mice was possible thanks to the cloning of Sig-1R, becoming in a very useful tool to study the biochemical and functional characteristics of the Sig-1R and to understand the *in vivo* role of this receptor (Langa et al., 2003).

2.2. Sig-1R: anatomical distribution, subcellular location and chaperoning function

The Sig-1R is expressed in anatomical areas important for pain control within the peripheral and the central nervous systems. In the peripheral nervous system these receptors are located in the dorsal root ganglion (DRG) (Sánchez-Fernández et al., 2014a), specifically in the soma of peripheral sensory neurons (Bangaru et al., 2013; Mavlyutov et al., 2016; Montilla-Garcia et al., 2018), and in Schwann cells (Palacios et al., 2004; Kwon et al., 2015b). In the central nervous system, Sig-1R are located in both the spinal cord dorsal horn and supraspinal sites (i.e. rostroventral medulla, periaqueductal gray matter and locus coeruleus) (Roh et al., 2008; Sánchez-Fernández et al., 2014a). It should be noted that the expression of Sig-1R is much higher in the DRG than in any area of the central nervous system tested (Sánchez-Fernández et al., 2014a), and this could indicate that Sig-1R could have a key role in the peripheral nervous system.

In addition to pain-related areas, Sig-1R are also located in regions related to memory and in structures of the limbic system, suggesting that they have an important role on brain plasticity and particularly in learning and memory processes (Bermack and Debonnel, 2005; Maurice et al., 2006; Chevallier et al., 2011). Sig-1R do not only exist in nervous system, but are also expressed in peripheral organs such as gastrointestinal tract, kidney, heart, liver and spleen, although its function in these areas has been less studied (Bowen, 2000; Stone et al., 2006; Bhuiyan and Fukunaga, 2011).

At the subcellular level, the Sig-1R is localized in multiple biological membranes, including plasma, nuclear, microsomal and mitochondrial membranes (Cobos et al., 2007). Sig-1R are particularly enriched in mitochondrion-associated endoplasmic reticulum (ER) membranes (MAM) (Hayashi and Su, 2007) (Figure K).

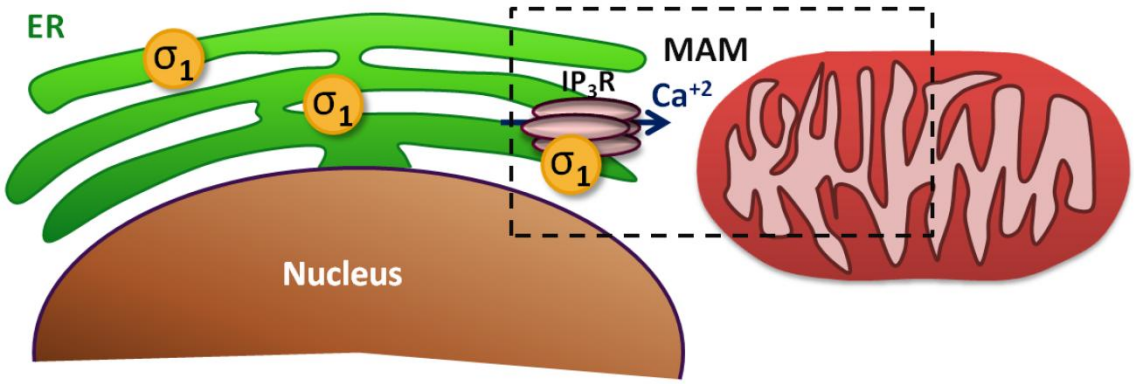

Figure K. Sigma-1 (σ1) receptors are located at mitochondrion-associated endoplasmic reticulum (ER) membranes (MAM). The Ca2+ influx from the ER to mitochondria is potentiated by Sig-1R. (Figure taken from Sánchez-Fernández, 2014b)

Under stress situations *in vitro*, the Sig-1R is translocated from the endoplasmic reticulum to the plasma membrane (Su et al., 2010), where it can interact with several protein targets, modifying their biological function (Su et al., 2016). Sig-1R agonists *in vitro* mimic the stressful response, with the consequent translocation of Sig-1R (Hayashi and Su, 2003). The interaction between Sig-1R and their protein targets is Ca^{2+}-dependent (Rodríguez-Muñoz et al., 2015a), and this suggests that Sig-1R act as an intracellular Ca^{2+} sensor to modulate neuronal physiology (Figure K).

Sig-1R have a chaperone domain at the C-terminus (residues 112-223) that lets it to bind to several target proteins and modify their functions (Hayashi and Su, 2007). The membrane targets of Sig-1R include several G-protein-coupled receptors (GPCR) and ion channels. Among the GPCRs that are targeted by Sig-1R are dopamine (D1 and D2) receptors (Rodriguez-Muñoz et al., 2015a), cannabinoid receptor 1 (CB1R) (Sánchez-Blazquez et al., 2014), serotonin receptors 1A (5-HT1AR) and 2A (5-HT2AR) (Rodriguez-Muñoz et al., 2015a), and μ-opioid receptors (MOR) (Kim et al., 2010; Rodriguez-Muñoz et al., 2015a; Rodriguez-Muñoz et al., 2015b). The ion channels that interact with Sig-1R are voltage-dependent K^+ channels ($K_v1.2$, $K_v1.3$, $K_v1.4$ and $K_v1.5$) (Kourrich et al., 2012; Kourrich et al., 2013), L-type voltage-dependent Ca^{2+} channels (VDCC) (Tchedre et al., 2008), voltage-gated sodium channels ($Na_v1.5$) (Balasuriya et al., 2012) acid-sensing ion channels of the 1a subtype (ASIC1a), gamma-aminobutyric acid type A ($GABA_A$) receptors (Su et al., 2010; Kourrich et al., 2012), and N-methyl-D-aspartate receptors (NMDARs) (Rodriguez-Muñoz et al., 2015a; Rodriguez-Muñoz et al., 2015b).

MOR and NMDAR are the protein targets of the Sig-1R which have been more deeply studied (reviewed by Sánchez-Fernández et al., 2017) (Figure L). Interestingly, some of these proteins not only can associate to Sig-1R, but they can also associate between themselves and act coordinately. For example, the function of μ-opioid receptors can be regulated by a complex formed by MOR, NMDAR and Sig-1R (Rodriguez-Muñoz et al., 2015a; Rodriguez-Muñoz et al., 2015b).

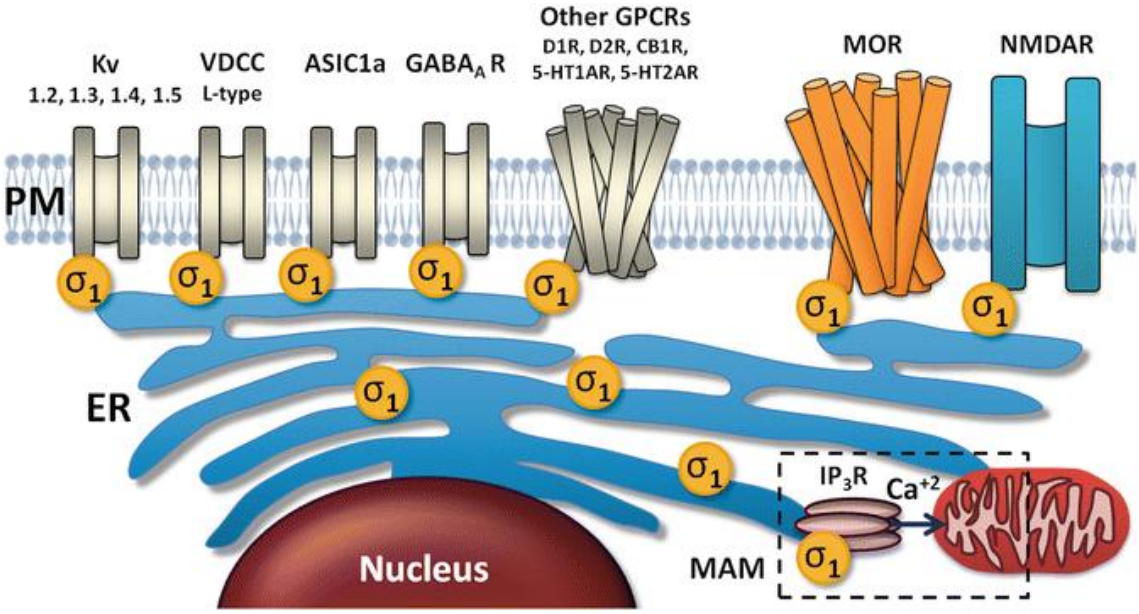

Figure L. Main protein targets of Sig-1R. Sigma-1 (σ_1) receptors located in the endoplasmic reticulum (ER) can modulate several receptors and channels in the plasma membrane (PM). The two best known protein targets of Sig-1R are the μ-opioid receptor (MOR) and the N-methyl-D-aspartate receptor (NMDAR), and they are represented at a larger scale for clarity. Other protein partners of Sig-1R at the PM, represented from left to right in the figure, include voltage-gated K^+ channels (Kv1.2, 1.3, 1.4, 1.5), L-type voltage-dependent calcium channels (VDCC), acid-sensing ion channels of the 1a subtype (ASIC1a), $GABA_A$ receptors ($GABA_AR$), and other G-protein- coupled receptors (GPCRs) such as dopamine (D1 and D2) receptors, cannabinoid receptor 1 (CB1R) and serotonin receptors 1A (5-HT1AR) and 2A (5-HT2AR). *(Figure taken from Sánchez-Fernández et al., 2017).*

2.3. Pharmacology of Sig-1R

The function of the Sig-1R can be regulated by synthetic and endogenous ligands. Endogenous ligands of Sig-1R are several neurosteroids (e.g. progesterone, pregnenolone, allopregnanolone, dehydroepiandrosterone…) (Cobos et al., 2008; Su et al., 2010) and the natural hallucinogen N,N-dimethyltryptamine (Fontanilla et al., 2009). However, although all these substances show affinity for Sig-1R, they are not selective for this receptor.

Sig-1R do not bind most opioid drugs; nevertheless, they bind, with high to moderate affinity, to a wide spectrum of known compounds with different pharmacological applications, such as antipsychotics (e.g. haloperidol), antidepressants (e.g. fluvoxamine), antitussives (e.g. carbetapentane), drugs for the treatment of neurodegenerative disorders (donepezil), and drugs of abuse (cocaine and methamphetamine) among others (Cobos et al., 2008; Su et al., 2010).

In addition, there are several selective Sig-1R agonists such as (+)-pentazocine and PRE-084, and Sig-1R antagonists such as BD-1047, NE-100, BD-1063 and S1RA (Cobos et al., 2008; Romero et al. 2012), which have been extensively employed to study the Sig-1R function. One of these compounds called S1RA has proved its selectivity for Sig-1R on a panel of 170 targets (Romero et al., 2012), and it has been extensively evaluated in pre-clinical models of chronic pain and recently in a Phase II clinical trial, showing efficacy in reducing chemotherapy-induced neuropathic pain with a good safety and tolerability profiles (Bruna et al., 2018; Bruna and Velasco, 2018). S1RA is the first Sig-1R ligand developed with an intended indication for neuropathic pain treatment.

2.4. Modulation of chronic pain by Sig-1R

There are substantial evidences that the Sig-1R is able to ameliorate pain states when the nociceptive system is sensitized, as it will be described in the present section.

Although the modulation of opioid antinociception by Sig-1R had been reported in the early to mid-1990s (reviewed by Sánchez-Fernández et al., 2017), the first evidence for the role of Sig-1R "per se" in pain modulation (in the absence of opioid drugs) was described a decade later in a model of chemically-induced pain (the formalin test) in mice (Cendán et al., 2005b). Intraplantar administration of formalin to rodents induces a biphasic pain response: an initial acute pain induced by direct nociceptor activation (first phase), followed by a tonic longer response (second phase), which is due to central sensitization (Le Bars et al., 2001). Systemic administration of Sig-1R antagonists (such as haloperidol or S1RA) was able to abolish both pain phases induced by formalin (Cendán et al., 2005a; Romero et al., 2012; Lan et al., 2014; Vidal-

Torres et al., 2014). Comparable results had already been obtained in Sig-1R-KO mice previously (Cendán et al., 2005b), indicating the specificity of the antinociceptive effects of the Sig-1R inhibition in the formalin test model. The amelioration of formalin-induced pain with the Sig-1R antagonist BD-1047 was associated with diminished phosphorylation of the NR1 subunit of NMDA receptors, which play a key role on central sensitization (Kim et al., 2006). Moreover, the administration of S1RA reduced the formalin-induced glutamate release and raised the noradrenaline levels in the spinal cord. Interestingly, the effect of Sig-1R antagonism on formalin pain was reverted by α_2-adrenoreceptor antagonism, indicating that the modulation of the descending noradrenergic system plays an important role in the effects of Sig-1R inhibition in the formalin-induced pain (Vidal-Torres et al, 2014). On the other hand, the local administration of S1RA into the formalin-injected paw also decreased formalin-induced pain (Vidal-Torres et al., 2014), indicating that Sig-1R can also modulate pain transmission locally.

The model of intraplantar administration of capsaicin was also used to study the role of Sig-1R in pain. The intradermal administration of capsaicin, that binds to TRPV1 and produces a strong C-fiber activation, produces mechanical hypersensitivity in the normal skin surrounding the injection place (named area of secondary hypersensitivity), due to a central sensitization state (Baron, 2000). In addition, capsaicin-induced mechanical hypersensitivity has a high translatability, as is performed in humans volunteers to test novel analgesics (Gottrup et al., 2004), and it is considered a surrogate model of neuropathic pain (Binder, 2016). Capsaicin-induced secondary hypersensitivity in mice was abolished after administration of several Sig-1R antagonists (S1RA, haloperidol, BD-1063, BD-1047 and NE-100) and in Sig-1R-KO mice (Entrena et al., 2009a and Entrena et al., 2009b; Romero et al., 2012), whereas Sig-1R agonists [PRE-084 and (+)-pentazocine] enhanced the hypersensitivity induced by capsaicin (Entrena et al., 2016).

It should be noted that the administration of the opioid antagonist naloxone did not reverse the effect of Sig-1R antagonism (by haloperidol or S1RA) on formalin and capsaicin tests (Cendán et al., 2005a; Entrena et al., 2009a; Vidal-Torres et al., 2014).

Therefore, the opioidergic system does not modulate the effects of Sig-1R in these pain models.

The capsaicin administration is also useful to produce visceral pain after its administration into the colon. Capsaicin-induced visceral pain shows two distinct components - intense (acute) pain and mechanical hyperalgesia referred to the abdominal wall (Laird et al., 2001). Sig-1R-KO mice displayed a decrease in the acute pain-like behaviors induced by intracolonic capsaicin, but did not show any amelioration of referred hyperalgesia in comparison to WT mice. However, the systemic administration of different selective Sig-1R antagonists (BD-1063, S1RA and NE-100) attenuated both acute pain-related behaviors and referred mechanical hyperalgesia induced by intracolonic capsaicin (González-Cano et al., 2013), suggesting the development of compensatory mechanisms in Sig-1R-KO mice.

The role of Sig-1R have also been studied in inflammatory pain. This kind of pain is characterized by the release of pro-inflammatory mediators, such as prostaglandins, cytokines, chemokines, proteases, neuropeptides, and growth factors at the site of inflammation, that are able to sensitize peripheral neurones producing pain (Muley et al., 2016). Using standard models of inflammatory pain (intraplantar administration of carrageenan, complete Freund´s adjuvant [CFA] or zymosan) it was reported that the systemic administration of several Sig-1R antagonists (S1RA, BD-1063, BD-1047 and MR200), ameliorated inflammatory mechanical and heat hypersensitivity in mice (Gris et al., 2014; Parenti et al., 2014a; Parenti et al., 2014b; Tejada et al., 2014; Jeong et al., 2015). It should be noted that Sig-1R-KO mice did not develop mechanical hyperalgesia after the administration of carrageenan (Tejada et al., 2014). However, the lack of Sig-1R was not able to reduce the heat hyperalgesia in inflamed animals (Gris et al., 2014; Tejada et al., 2014). These results with Sig-1R-KO mice which are in contrast with those found with Sig-1R antagonists, may be explained by the development of compensatory mechanisms in mutant mice (Sánchez-Fernández et al., 2017).

2.5. Role of Sig-1R in neuropathic pain

The role of Sig-1R has also been studied in different models of neuropathic pain in mice and rats, most of them, models of peripheral neuropathic pain (see table 1). Some of the most used models to study the antineuropathic effect of Sig-1R inhibition are those induced by a mechanical peripheral nerve injury, such as the partial sciatic nerve ligation (PSNL) model (de la Puente et al., 2009; Romero et al., 2012), chronic constriction of the infraorbital nerve (a model of trigeminal neuropathic pain) (Gris et al., 2016), chronic constriction injury (CCI) of sciatic nerve model (Moon et al., 2015; García-Martínez et al., 2016; Kang et al., 2016; Choi et al., 2017) and chronic compression of dorsal root ganglia (CCD) model (Son and Kwon, 2010) (Table 1). In addition, Sig-1R inhibition is also effective inhibiting pain-like behaviors in models of diabetic neuropathy (Gris et al., 2016; Paniagua et al., 2017; Wang et al., 2018), bone cancer pain (Zhu et al., 2015) and neuropathic pain induced by antineoplastics, such as paclitaxel (Nieto et al., 2012; Nieto et al., 2014), oxaliplatin (Gris et al., 2016), cisplatin (Paniagua et al., 2019) or vincristine (Paniagua et al., 2019) (Table 1).

Mechanical hypersensitivity associated to PSNL model (Díaz et al., 2012; Romero et al., 2012; Bura et al., 2013), trigeminal neuropathic pain (Gris et al., 2016), CCI model (Kang et al., 2016; Espinosa-Juárez et al., 2017a; Espinosa-Juárez et al., 2017b), CCD model (Son and Kwon, 2010), chemotherapy-induced peripheral neuropathy (CIPN) (Nieto et al., 2012; Gris et al., 2016; Paniagua et al., 2019), diabetic neuropathy (Gris et al., 2016; Paniagua et al., 2017) and bone cancer pain (Zhu et al., 2015) was reverted by the systemic acute administration of several selective Sig-1R antagonists (S1RA, BD-1047, BD-1063) and some new drugs proposed as Sig-1R antagonists (NMIN, I-TPH) (Table 1).

Acute systemic administration of Sig-1R antagonists (S1RA, BD-1063, BD-1047, NMIN) to WT animals, also ameliorated cold allodynia induced by PSNL model (Bura et al., 2013), CCI model (García-Martínez et al., 2016; Espinosa-Juárez et al., 2017a), CCD model (Son and Kwon, 2010) and CIPN (Nieto et al., 2012; Gris et al., 2016) (Table 1).

The effects of Sig-1R antagonist on heat hypersensitivity have been less studied, although it has been reported that the acute systemic treatment with S1RA reverted

the heat hyperalgesia induced by PSNL model (Díaz et al., 2012; Romero et al., 2012; Bura et al., 2013) and diabetic neuropathy (Paniagua et al., 2017) (Table 1).

Interestingly, systemic pharmacological antagonism of Sig-1R was not only able to reverse neuropathic hypersensitivity once the neuropathy was developed, but it was also effective in attenuating its development when the Sig-1R antagonists were given repeatedly for several days during the induction of the neuropathy. Thus, the administration of different Sig-1R antagonists (S1RA, BD-1047 and BD-1063) reduced the development of mechanical (Son and Kwon, 2010; Nieto et al., 2012; Romero et al., 2012; Nieto et al., 2014; Gris et al., 2016; Paniagua et al., 2017; Paniagua et al., 2019), cold (Son and Kwon, 2010; Nieto et al., 2012; Romero et al., 2012; Nieto et al., 2014; Gris et al., 2016) and heat hypersensitivity (Romero et al., 2012; Paniagua et al., 2017) in different models of peripheral neuropathic pain (Table 1). The ameliorative effects displayed by the repeated treatment with Sig-1R antagonists disappeared after treatment was discontinued, and did not induce tolerance to the antihypersensitivity effects (Son and Kwon, 2010; Romero et al., 2012).

Some authors administered Sig-1R antagonists intrathecally showing that the acute injection of BD-1047 or I-THP attenuated mechanical allodynia induced by CCI (Kang., 2016) and bone cancer (Zhu et al., 2015) (Table 1). In addition, repeated intrathecal administration of the Sig-1R antagonist BD-1047 suppressed CCI-induced mechanical allodynia but not heat hyperalgesia when this antagonist was given during the induction phase of neuropathic pain (days 0-5 after CCI surgery) but it did not have any effect when administered during the maintenance phase (days 15-20 after CCI surgery) (Roh et al., 2008, Choi et al., 2013; Moon et al., 2013; Moon et al., 2014; Moon et al., 2015; Choi et al., 2017) (Table 1). Furthermore, the ameliorative effect of the repeated intrathecal administration of BD-1047 resulted in a sustained effect since it did not disappear after treatment was discontinued (Roh et al., 2008; Moon et al., 2014; Moon et al., 2015) (Table 1). In contrast, the repeated intrathecal administration of BD-1047 (during 7 days) attenuated not only mechanical hypersensitivity, but also heat hypersensitivity induced by diabetic neuropathy (Table 1) (Wang et al., 2018).

The role of Sig-1R in neuropathic pain has also been explored in mutant mice lacking Sig-1R (Table 1). Similar to the effect obtained with the pharmacological inhibition of the Sig-1R, mechanical or/and cold allodynia were attenuated in Sig-1R-KO mice, in a variety of models of neuropathic pain (de la Puente., 2009; Nieto et al., 2012; Nieto et al., 2014; Song et al., 2017; Wang et al., 2018). However, controversial results with Sig-1R-KO animals were reported as they developed a similar heat hyperalgesia than that of wild-type (WT) mice in the PSNL model (de la Puente et al., 2009), whereas this sensory modality was attenuated in Sig-1R-KO mice in diabetic neuropathy (Wang et al., 2018) (Table 1). Therefore, there seems to be a mismatch between the effect of pharmacological and genetic inhibition of Sig-1R on heat hypersensitivity (De la Puente et al., 2009; Romero et al., 2012), which was also found during inflammatory pain (Tejada et al. 2014) and that might be attributed to unknown compensatory mechanisms developed by Sig-1R-KO mice (Sánchez-Fernández et al., 2017).

In addition, the selective Sig-1R antagonist S1RA ameliorated anhedonia associated to neuropathic pain (measured as a decreased preference for a sweetened liquid) (Bura et al., 2013) (Table 1). Anhedonia is the lack of interest or pleasure in responding to normally pleasurable stimuli or experiences that can be induced by the neuropathic pain condition (Cobos and Portillo-Salido, 2013). This result suggests that Sig-1R antagonists could be useful not only as a drug to treat neuropathic pain but also to improve some emotional changes during pain states.

The role of Sig-1R has also been studied in central neuropathic pain, in particular, in the model of spinal cord contusion injury (SCI) (Choi et al., 2016; Castany et al., 2018; Castany et al., 2019). The acute systemic treatment with the Sig-1R antagonists BD-1047 or S1RA was able to reverse neuropathic hypersensitivity once the neuropathy was developed (Castany et al., 2018), and to attenuate its development when the Sig-1R antagonists where administrated repeatedly (Choi et al., 2016; Castany et al., 2019). Furthermore, the mechanical allodynia and heat hyperalgesia was attenuated in Sig-1R-KO mice in this model (Castany et al., 2018).

Table 1: Role of Sig-1R in different neuropathic pain models.
Summary of the effects of Sig-1R inhibition on neuropathic pain models by the systemic or intrathecal administration of Sig-1R antagonists in wild-type animals (mice or rats) or in Sig-1R knockout mice. Mechanical allodynia was assessed with von Frey filaments, cold allodynia was evaluated with a cold plate or acetone test, and heat hyperalgesia was assessed with a Hargreaves device or a hot plate. Sig-1R knockout are classified as "preventive" treatment, since Sig-1R inhibition was present before the peripheral nerve injury. The pharmacological treatment with the selective Sig-1R antagonists was administrated once (acute) or more times (repeated).

Injury	Readout	Sig-1R antagonist/ Sig-1R-KO	Route	Prevented/ Acute/ Repeated	Effect on allodynia/ hyperalgesia	Mouse/ rat	References
PSNL	Mechanical allodynia	Sig-1R-KO	-	Prevented	Attenuation	mouse	de la Puente et al., 2009
		S1RA	Systemic	Repeated	Attenuation	mouse	Romero et al., 2012
			Systemic	Acute			Díaz et al., 2012; Romero et al., 2012; Bura et al., 2013
	Cold allodynia	Sig-1R-KO	-	Prevented	Attenuation	mouse	de la Puente et al., 2009
		S1RA	Systemic	Repeated	Attenuation	mouse	Romero et al., 2012
				Acute			Bura et al., 2013
	Heat hyperalgesia	Sig-1R-KO	-	Prevented	No effect	mouse	de la Puente et al., 2009
		S1RA	Systemic	Repeated	Attenuation	mouse	Romero et al., 2012
				Acute			Díaz et al., 2012; Romero et al., 2012; Bura et al., 2013
	Anhedonia	S1RA	Systemic	Acute	Attenuation	mouse	Bura et al., 2013
Trigeminal neuropathic pain	Mechanical allodynia	S1RA	Systemic	Acute	Attenuation	rat	Gris et al., 2016
				Repeated			
CCI	Mechanical hyperalgesia	NMIN	Systemic	Acute	Attenuation	rat	Espinosa-Juárez et al., 2017a; Espinosa-Juárez et al., 2017b
		BD-1063					
	Mechanical allodynia	BD-1047	i.t.	Repeated	Attenuation*	rat	Roh et al., 2008, Choi et al., 2013; Moon et al., 2013; Moon et al., 2014; Moon et al., 2015; Choi et al., 2017
				Acute	Attenuation	mouse	Kang et al., 2016
		I-THP	Systemic	Acute	Attenuation	mouse	Kang et al., 2016
			i.t.				Kang et al., 2016
	Heat hyperalgesia	BD-1047	i.t.	Repeated	No effect	rat	Roh et al., 2008; Choi et al., 2013; Moon et al., 2013; Moon et al., 2014; Moon et al., 2015; Choi et al., 2017
	Cold allodynia	BD-1063	Systemic	Acute	Attenuation	rat	Espinosa-Juárez et al., 2017a; García-Martínez et al., 2016
		NMIN					García-Martínez et al., 2016

Injury	Readout	Sig-1R antagonist/ Sig-1R-KO	Route	Prevented/ Acute/ Repeated	Effect on allodynia/ hyperalgesia	Mouse/ rat	References
CCD	Mechanical allodynia	BD-1047	Systemic	Repeated	Attenuation	rat	Son and Kwon, 2010
				Acute			
	Cold allodynia			Repeated			
				Acute			
Peripheral Neuropathy Induced by Paclitaxel	Mechanical allodynia	Sig-1R-KO	-	Prevented	Attenuation	mouse	Nieto et al., 2012; Nieto et al., 2014
		S1RA	Systemic	Repeated	Attenuation	mouse	Nieto et al., 2012
				Acute			
		BD-1063	Systemic	Repeated	Attenuation	mouse	Nieto et al., 2012; Nieto et al., 2014
				Acute			Nieto et al., 2012
	Cold allodynia	Sig-1R-KO	-	Prevented	Attenuation	mouse	Nieto et al., 2012; Nieto et al., 2014
		S1RA	Systemic	Repeated	Attenuation	mouse	Nieto et al., 2012
				Acute			
		BD-1063	Systemic	Repeated	Attenuation	mouse	Nieto et al., 2012; Nieto et al., 2014
				Acute		mouse	Nieto et al., 2012
Peripheral Neuropathy Induced by cisplatin	Mechanical allodynia	S1RA	Systemic	Repeated	Attenuation	rat	Paniagua et al., 2019
				Acute			
	Mechanical hyperalgesia	S1RA	Systemic	Acute	Attenuation	rat	Paniagua et al., 2019
Peripheral Neuropathy Induced by oxaliplatin	Cold allodynia	S1RA	Systemic	Acute	Attenuation	rat	Gris et al., 2016
				Repeated			Gris et al., 2016
Peripheral Neuropathy Induced by vincristine	Mechanical allodynia	S1RA	Systemic	Repeated	Attenuation	rat	Paniagua et al., 2019
				Acute			
	Mechanical hyperalgesia	S1RA	Systemic	Repeated	Attenuation	rat	Paniagua et al., 2019
				Acute			
Diabetic neuropathy	Mechanical hyperalgesia	S1RA	Systemic	Acute	Attenuation	rat	Gris et al., 2016
				Repeated			
	Mechanical allodynia	Sig-1R-KO	-	Prevented	Attenuation	mouse	Song et al., 2017; Wang et al., 2018
		BD-1047	i.t.	Repeated	Attenuation	mouse	Wang et al., 2018
		S1RA	Systemic	Acute	Attenuation	rat	Paniagua et al., 2017
				Repeated			
	Heat hyperalgesia	Sig-1R-KO	-	Prevented	Attenuation	mouse	Wang et al., 2018
		BD-1047	i.t.	Repeated	Attenuation	mouse	Wang et al., 2018
		S1RA	Systemic	Acute	Attenuation	rat	Paniagua et al., 2017
				Repeated			Paniagua et al., 2017
	Heat hypoalgesia	Sig-1R-KO	-	Prevented	Attenuation	mouse	Song et al., 2017
Bone Cancer Pain	Mechanical allodynia	BD-1047	i.t.	Acute	Attenuation	rat	Zhu et al., 2015
SCI	Mechanical allodynia	Sig-1R-KO	-	Prevented	Attenuation	mouse	Castany et al., 2018
		BD-1047	Systemic	Repeated	Attenuation *		Choi et al., 2016
		S1RA	Systemic	Repeated	Attenuation		Castany et al., 2019
				Acute			Castany et al., 2018
	Heat hyperalgesia	Sig-1R-KO	-	Prevented	Attenuation	mouse	Castany et al., 2018
		S1RA	Systemic	Repeated			Castany et al., 2019
				Acute			Castany et al., 2018

*Attenuation during the induction phase (days 0-5 after CCI surgery) but not during the maintenance phase (days 15-20 after CCI surgery).

CCD: chronic compression of dorsal root ganglion, CCI: chronic constriction injury, i.p. intraperitoneal, i.t. intrathecal, i.v. intravenous, Sig-1R-KO: sigma-1 receptor knockout, PSNL: partial sciatic nerve ligation, s.c. subcutaneous, SCI: spinal cord contusion injury, SNI: spared nerve injury

In addition to the modulation of neuropathic pain-like behaviors, it has been described that Sig-1R can regulate different cellular events associated to neuropathic pain states. Thus, the own Sig-1R expression was upregulated in the dorsal spinal cord after peripheral nerve injury (Roh et al., 2008; Choi et al., 2013). The NMDA receptor, which has a key role in the central sensitization process that take place during neuropathic pain (Latremoliere and Woolf, 2009), is one of the main protein target which are regulated by the Sig-1R (reviewed by Sánchez-Fernández et al., 2017). The blockade of Sig-1R decreased the phosphorylation of the NR1 and/or NR2B subunits of NMDA receptors after SCI (Castany et al., 2018; Castany et al., 2019), diabetic neuropathy (Song et al., 2017), CCI (Roh et al., 2008; Kang et al., 2016) and bone cancer pain (Zhu et al., 2015) (Table 2).

In addition, Sig-1R inhibition also decreases the activation in the spinal cord of protein kinases, such as ERK and p38 Mitogen-Activated Protein Kinase [p38 MAPK], which are important players for central sensitization during neuropathic pain. The increase in the phosphorylation of extracellular signal-regulated kinase (ERK1/2) during SCI (Castany et al., 2018; Castany et al., 2019), PSNL (de la Puente et al., 2009), chemotherapy-induced peripheral neuropathy (Nieto et al., 2012), CCD model (Son and Kwon, 2010) and bone cancer pain (Zhu et al., 2015) was reduced in animals with Sig-1R blockade (Table 2). Moreover, CCI and bone cancer pain produced phosphorylation of p38 MAPK that was attenuated after the treatment with the selective antagonist BD-1047 (Moon et al., 2013; Moon et al., 2014; Zhu et al., 2015).

It has also been described that the Sig-1R could have an important role in modulating some pro-inflammatory mediators during chronic pain. Thereby, Sig-1R might have an important role modulating the changes in the expression and intracellular distribution of the proposed proinflammatory mediator of chronic pain HMGB1 (High-mobility group Box 1) during diabetic neuropathy (Wang et al., 2018) (Table 2). Furthermore, Sig-1R inhibition decreased the expression of the pro-inflammatory cytokines TNF-α and IL-1β (Zhu et al., 2015; Castany et al., 2018; Castany et al., 2019) as well as the activation of astrocytes and microglia in the spinal cord, which play an important role on central sensitization (Moon et al., 2014; Moon et al., 2015; Zhu et al., 2015; Choi et al., 2016) (Table 2).

On the other hand, WT mice treated systemically with the Sig-1R antagonist BD-1063 and Sig-1R-KO mice showed a decrease in paclitaxel-induced mitochondrial abnormalities (as a sign of neurotoxicity induced by the chemotherapeutic drug) in myelinated A-fibers (Nieto et al., 2014) (Table 2). Interestingly, a different research group showed later that A-δ fibers were hyperactivated after the treatment with others antineoplastics, such as vincristrine and cisplatin, as well as in diabetic neuropathy, and this A-δ fibers hyperactivity was reduced with the treatment with S1RA in all cases (Paniagua et al., 2017; Paniagua et al., 2019) (Table 2).

Finally, the role of Sig-1R in the generation of reactive oxygen species (ROS) has also been studied. ROS have been suggested to have an important involvement in neuropathic pain, and they have also been implicated in NMDA receptor activation (Kim et al., 2004). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) is an enzyme system that generates ROS and its activation as well as the ROS production in the spinal cord were attenuated after the administration of the Sig-1R antagonist BD-1047 (Choi et al., 2013).

Therefore, Sig-1R have an important role in the development and maintenance of neuropathic pain and it has been described that it is implicated in some mechanisms related to chronic pain. In fact, it has been proposed that Sig-1R is essential for central sensitization (Drews and Zimmer, 2009). Furthermore, taking into consideration that the expression of Sig-1R is much higher in the DRG than in any central area tested and that the participation of Sig-1R in neuropathic pain has been studied very little at peripheral level, further studies at peripheral level are warranted.

Table 2: Role of Sig-1R in neuropathic pain-mediated changes.
The table summarizes the effect of Sig-1R inhibition on the neurochemical and histopathological changes associated to neuropathic pain of diverse etiology.

Neuropathic pain-mediated changes		Injury	Sig-1R-KO / drug	mouse / rat	References
Reduction of the increase of central sensitization markers	Decreased activation/ phosphorylation of NMDA receptors	SCI (1)	Sig-1R-KO	mouse	Castany et al., 2018
		Diabetic neuropathy (2)	Sig-1R-KO		Song et al., 2017
		CCI (3)	BD-1047		Kang et al., 2016
				Rat	Roh et al., 2008
		Bone cancer pain (3)			Zhu et al., 2015
		SCI (1)	S1RA	mouse	Castany et al., 2019
	Decreased activation/ phosphorylation of ERK1/2	SCI	Sig-1R-KO	mouse	Castany et al., 2018
		PSNL			de la Puente et al., 2009
		Chemotherapy-induced peripheral neuropathy			Nieto et al., 2012
		SCI	S1RA		Castany et al., 2019
		CCD	BD-1047	Rat	Son and Kwon, 2010
		Bone cancer pain			Zhu et al., 2015
	Reduced phosphorylation of p-p38 MAPK	CCI	BD-1047	rat	Moon 2013; Moon 2014
		Bone cancer pain			Zhu 2015
Reduction of the increase of HMGB1		Diabetic neuropathy	Sig-1R-KO	mice	Wang et al., 2018
Reduction of the increase of proinflamatory citokines (TNF- α or/and IL-1β)		SCI	Sig-1R-KO	mouse	Castany et al., 2018
			S1RA		Castany et al., 2019
		Bone cancer pain	BD-1047	rat	Zhu et al., 2015
Reduction of microglia activation		Bone cancer pain	BD-1047	rat	Zhu et al., 2015
Reduction of astrocyte activation		SCI	BD-1047	mouse	Moon et al., 2014; Choi et al., 2016
Reduction of the increase of d-serine and Srr in astrocytes		CCI	BD-1047	Rat	Moon et al., 2015
Reduction of mitochondrial abnormalities		Chemotherapy-induced peripheral neuropathy	Sig-1R-KO	mouse	Nieto et al., 2014
			BD-1063		
Reduction of electrophysiological hyperactivity of Aδ-primary afferents		Diabetic neuropathy	S1RA	rat	Paniagua et al., 2017
		Chemotherapy-induced peripheral neuropathy			Paniagua et al., 2019
Reduction of NADPH oxidase 2 activation and ROS production.		CCI	BD-1047	Rat	Choi et al., 2013

(1) Decreased activation/phosphorylation of NR2B-NMDA. (2) Decreased activation/phosphorylation of subunits GluN2A and GluN2B of NMDARs. (3) Decreased activation/phosphorylation of subunit NR1 of NMDARs. CCI: chronic constriction injury, ERK: extracellular signal-regulated kinase, HMGB1: high-mobility group box 1, IL1β: interleukin 1β, Sig-1R-KO: Sig-1R knockout, NADPH: nicotinamide adenine dinucleotide phosphate, NMDA: N-Methyl-D-Aspartate, p38: p38 mitogen-activated protein kinase, PSNL: partial sciatic nerve ligation, ROS: reactive oxygen species, SCI: spinal cord contusion injury, SNI: spared nerve injury, Srr: serine racemase, TNF-α: tumor necrosis factor-alpha.

2.6. Immune cells and peripheral opioid analgesia: role of Sig-1R

Numerous studies have reported that both central and peripheral Sig-1R inhibition enhances opioid antinociception induced by administration of opioid receptor agonist drugs (Mei and Pasternak, 2002; Marrazo et al., 2006; Mei and Pasternak, 2007; Sánchez-Fernández et al., 2013; Sánchez-Fernández et al., 2014a; Montilla-García et al., 2018; Montilla-García et al., 2019). However, few studies have evaluated whether Sig-1R inhibition also modulates endogenous opioid effects.

During an inflammatory process, immune cells infiltrate into the inflamed tissue and release proalgesic agents that sensitize nociceptors (Hoebe et al., 2004; Mahdavian et al., 2011; Ji et al., 2014; Ghasemlou et al., 2015) (Figure M). These immune cells also produce endogenous opioid peptides (EOPs) with analgesic potential (Hua and Cabot, 2010; Sauer et al., 2014; Wang et al., 2014) (Figure M). Therefore, during the inflammatory process, there is a myriad of pronociceptive and analgesic agents released by immune cells, although the balance between the analgesic and proalgesic effects of immune cells is normally shifted toward pronociception. Interestingly, it has been described that Sig-1R antagonism regulates the neuromodulatory actions of the immune system and shift the balance between the proalgesic/analgesic effects toward analgesia (Tejada et al., 2018). In fact, the Sig-1R inhibition enhanced the analgesic effect of EOPs released by neutrophils and macrophages after the inflammation produced by carrageenan administration (Tejada et al., 2017) (Figure M).

Interestingly, many studies have revealed that not only inflammation but also neuropathy can be associated with immune reactions (Labuz et al., 2016). After peripheral nerve lesion, resident cells (e.g., macrophages, Schwann cells, fibroblasts, microglia and astrocytes) are activated and leukocytes (e.g., neutrophils, monocytes/macrophages and T lymphocytes) are recruited to the site of injury and to other adjacent areas of the nervous system (Scholz and Woolf, 2007). As it was mentioned above, these immune cells can release opioid peptides that could ameliorate neuropathy-induced hypersensitivity (Labuz et al., 2009; Labuz et al., 2010; Liou et al., 2011; Tejada et al., 2018). Upregulation of EOPs in the DRG and spinal cord of rats, such as prodynorphin or pronociceptin, has been described in the CCI model.

This upregulation was attenuated with the systemic administration of minocycline, in parallel with a decrease in the microglial/macrophagic activation in the spinal cord and the trafficking of peripheral immune cells into the DRG (Mika et al., 2010). However, it is unknown whether Sig-1R curtail the antinociceptive effects mediated by EOPs during neuropathy, and thereby facilitate neuropathic pain, as it has been described in a model of inflammatory pain.

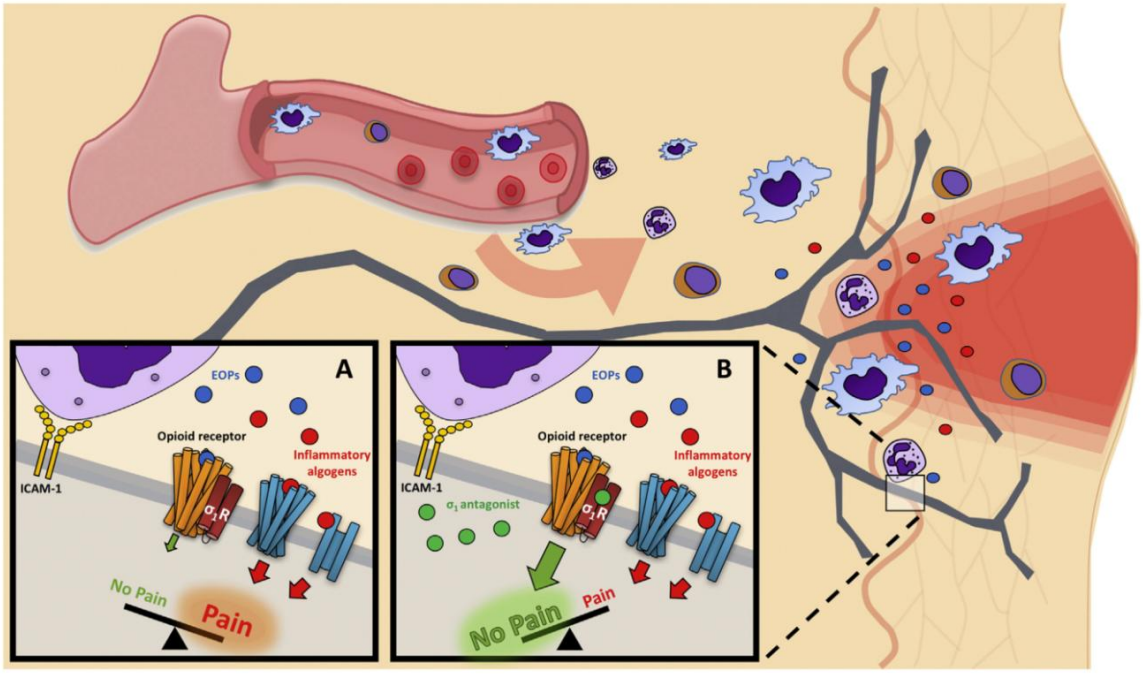

Figure M. Effect of Sig-1R antagonism on immune-driven opioid analgesia. Immune cells migrate to the inflamed tissue, where they interact with peripheral nerve terminals which express the intercellular adhesion molecule-1 (ICAM-1). These immune cells release both inflammatory algogens, which promote pain, and endogenous opioid peptides (EOPs). (A) Under normal conditions the overall result of this immune-neuron interaction is pain, because sigma-1 receptors (σ1-R) tonically inhibit opioid receptors, and hence decrease the analgesic effect of EOPs. (B) In the presence of a Sig-1R antagonist, the effects of opioid receptors are enhanced, potentiating the effects of EOPs of immune origin, and resulting in immune-driven opioid analgesia (Figure taken from Tejada et al., 2018).

RATIONALE AND GOALS

RATIONALE AND GOALS

Substantial preclinical evidence points to a prominent role for Sig-1R in pathological pain of diverse etiology since pain-like behaviors are attenuated in Sig-1R knockout mice (de la Puente et al., 2009; Nieto et al., 2012; Tejada et al., 2017) and in wild-type animals treated with Sig-1R antagonists (Roh et al., 2008; Son and Kwon, 2010; Nieto et al., 2012; Romero et al., 2012; Gris et al., 2016; Tejada et al., 2017). In addition, Sig-1R antagonists modify the chaperoning activity of these receptors by rising the endogenous opioidergic signaling and decreasing the activities of NMDA receptor and ERK, consequently decreasing the sensory hypersensitivity that characterizes pathological pain conditions (reviewed by Sánchez-Fernández et al., 2017). Consistent with these favorable results in animals, one of the selective Sig-1R antagonists available, called S1RA, has been evaluated in a Phase II clinical trial, with promising results for management of oxaliplatin-induced neuropathic pain (Bruna et al., 2018; Bruna and Velasco, 2018).

The role of the Sig-1R in neuropathic pain has been studied in neuropathies induced by chemotherapy, diabetes and by traumatic injury of peripheral nerves (De la Puente et al., 2009; Romero et al., 2012; Nieto et al., 2012; Paniagua et al., 2017; Paniagua et al., 2019). Nevertheless, all studies to date on the role of Sig-1R in neuropathic pain after mechanical injury to peripheral nerves have focused on models that involved the constriction or ligation of the sciatic nerve (Roh et al., 2008; De la Puente et al., 2009; Bura et al., 2013; Gris et al., 2016), but the role of these receptors in neuropathic pain induced by nerve transection has never been explored. Importantly, surgical interventions inevitably results in nerve transections, and as a consequence, significant number of patients experience neuropathic pain (Borsook et al., 2013). Moreover, there are differences between the pathophysiological mechanisms and response to drug treatment of neuropathic pain induced by different types of injury to peripheral nerves (Baron et al., 2010; Hershman et al., 2014; Finnerup et al., 2015). In particular, it is well stablished that the neuroplastic changes differ between models of nerve constriction and nerve section (Casal-Diaz et al., 2009); therefore, the **first goal** of this Doctoral Thesis was to evaluate whether Sig-1R inhibition ameliorates neuropathic pain induced by nerve transection. To this end, we used different experimental

approaches. We studied the effects of acute Sig-1R inactivation by the systemic administration of the Sig-1R antagonist S1RA on neuropathic pain-like behaviors in the spared nerve injury (SNI) model. In this model, the tibial and common peroneal branches of the sciatic nerve were ligated with a silk suture and transected distally, while the sural nerve was left intact. Moreover, we took advantage of the genetic approach using Sig-1R knockout mice to examine the effects of the genetic blockade of Sig-1R in SNI-induced neuropathic pain. Finally, in order to test the effect of prolonged pharmacological antagonism of Sig-1R on the development of neuropathic pain after nerve transection, we evaluated the effect of systemic repeated administration of S1RA on pain hypersensitivity induced by SNI.

Another potential indication of the antagonists of Sig-1R is the potentiation of opioid analgesia (Vela et al., 2015). In fact, Sig-1R physically interact with opioid receptors restraining their functioning (Sánchez-Fernández et al., 2017). Previous studies showed that Sig-1R inhibition is able to enhance antinociception induced by opioid drugs through both central (Mei and Pasternak, 2002) and peripheral (Sánchez-Fernández et al., 2014a) opioid receptors. In addition, it is known that Sig-1R inhibition enhances the analgesic effect of endogenous opioid peptides produced naturally by immune cells that accumulate at the inflamed site to relieve inflammatory pain (Tejada et al., 2017). However, whether Sig-1R modulates endogenous opioid analgesia in neuropathic pain remains completely unknown. Accordingly, the **second goal** of this Doctoral Thesis was to determine the contribution of endogenous opioid analgesia to the antineuropathic effects induced by Sig-1R antagonism, and, in particular, whether peripheral opioid receptors are involved in this process. In order to address this question, we tested whether the antineuropathic effects of S1RA could be reversed by both the opioid antagonist naloxone and its peripherally-restricted analog naloxone methiodide.

The mechanisms involved in the neuropathic pain relieve produced by the Sig-1R inhibition have been mainly studied at central nervous system level, where it is known to modulate central sensitization after peripheral nerve injury (Sánchez-Fernández et al., 2017). However, little is known about the contribution of peripheral Sig-1R to neuropathic pain, in spite of the fact that they are highly expressed in the dorsal root ganglia (DRG) (Sánchez-Fernandez et al., 2014a); where they are solely located in

sensory neurons and not in satellite glial cells or other cells (Mavlyutov et al., 2016; Montilla-Garcia et al., 2018). At subcellular level, the Sig-1R is mainly expressed at the mitochondrion-associated endoplasmic reticulum membranes (MAM) and, under cellular stress situations, this receptor can translocated to other areas of the cell, such as the plasma or nuclear membranes, where they can physically interact with different protein targets (Su et al., 2010; Tsai et al., 2015).

Peripheral nerve transection produces the degeneration and clearance of the distal nerve injured (Cheng et al., 2017), but also changes in the cell bodies of the axotomized DRG neurons (Zimmermann et al., 2001; Hu et al., 2016; Wiberg et al., 2018), like the expression of several transcription factors including ATF3 (Laedermann et al., 2014), which is a marked of cellular stress. Whether the section of a nerve that causes cellular stress in DRG neurons, also induces changes in the subcellular distribution of the Sig-1R (as those described above) in the soma of DRG neurons has never been explored, and in consequence, this question constitutes the **third goal** of this Doctoral Thesis. To this end, we performed immunohistochemistry experiments to study changes in the expression pattern of Sig-1R and cellular stress markers in neurons from the injured DRG in response to nerve transection.

It has been described that the Sig-1R has a role in the neuroinflammation associated to neuropathic pain at the spinal cord level. Thus, Sig-1R inhibition decreases the expression of proinflammatory cytokines (Zhu et al., 2015; Castany et al., 2018; Castany et al., 2019), the activation of ERK1/2 (de la Puente et al., 2009; Son and Kwon, 2010; Nieto et al., 2012) and NMDA receptors (Roh et al., 2008; Kang et al., 2016; Song et al., 2017), as well as the activity of astrocytes and microglia in the spinal cord (Moon et al., 2014; Zhu et al., 2015; Choi et al., 2016) during neuropathic pain. In response to peripheral nerve injury, DRG neurons can release the chemokine CCL2, which has a potent chemotactic activity recruiting macrophages into the DRG (Zhu et al., 2014). After nerve transection, macrophages invade the DRG (Kwon et al., 2013) and surround DRG neurons forming 'ring-like' structures (Vega-Avelaira et al., 2009). The recruitment of immune cells, such as peripheral macrophages, into the DRG plays an active role in neuropathic pain through the local release of proinflammatory algogenic mediators which can sensitize the neighbor neurons (Ji et al., 2016).

However, the role of Sig-1R in the neuroinflammation that takes place in the DRG during neuropathic pain has never been studied.

Taking into account these antecedents, our **fourth goal** was to evaluate the role of Sig-1R on macrophage recruitment into the DRG in neuropathic pain induced by nerve transection. To achieve this goal, we first studied by immunohistochemistry whether the changes in the distribution pattern of Sig-1R in DRG neurons after nerve transection have any role in the macrophage recruiting. In addition, using cell-specific markers, we evaluated the degree of infiltration of macrophages/monocytes into the DRG (in terms of number and location) after nerve transection, through fluorescence-activated cell sorting (FACS) and immunohistochemistry, in wild type and Sig-1R knockout mice. Finally, we also determine the role of Sig-1R in the release of the chemokine CCL2 in the DRG after nerve transection, through the quantification of CCL2 levels by enzyme-linked immunosorbent assay (ELISA), in wild-type and Sig-1R-KO mice.

EXPERIMENTAL STUDIES

EXPERIMENTAL STUDIES

1. STUDY 1: Sigma-1 receptor inhibition reduces neuropathic pain induced by partial sciatic nerve transection in mice by opioid-dependent and -independent mechanisms

1.1. Material and methods

1.1.1. Animals

Most experiments were performed in 8- to 11-week-old female WT CD-1 mice (Charles River, Barcelona, Spain) and Sig-1R-KO CD-1 mice (Laboratorios Esteve, Barcelona, Spain). Some experiments were performed on male mice from the same strain and genotypes. Taking into account that male mice are much more aggressive to other mice than female animals (Edwards, 1968), and that stress such us that induced by fights with the alpha male can induce opioid analgesia (Miczek et al., 1982), we considered that this behavior of male mice might be a confounder in our experiments in the context on the modulation of endogenous opioid analgesia by Sig-1R. Therefore, we performed most experiments in female mice. However, we also tested male mice in some key experiments (see the Results section) to explore a possible sexual dimorphism in Sig-1R mediated modulation of SNI-induced hypersensititivy. Female animals were tested at random times throughout the estrous cycle. Mice were housed in colony cages with free access to food and water prior to the experiments, and were kept in temperature- and light-controlled rooms (22 ± 2 °C, and light-dark cycle of 12 hours). The experiments were done during the light phase (from 9:00 a.m. to 3:00 p.m.). Animal care was in accordance with international standards (European Communities Council Directive 2010/63) and the protocol of the study was approved by the Research Ethics Committee of the University of Granada, Spain.

1.1.2. Spared nerve injury

Mice were anesthetized with isoflurane (3%), and SNI surgery was performed as previously described (Bourquin et al., 2006). Briefly, an incision was made in the left thigh skin and was followed by an incision made directly through the biceps femoris muscle, exposing the sciatic nerve and its three terminal branches (the sural, common peroneal and tibial nerves). The tibial and common peroneal branches of the sciatic nerve were ligated with a silk suture and transected distally, while the sural nerve was left intact. In sham-operated control mice, the sciatic nerve terminal branches were exposed but neither ligated nor transected. The day of SNI surgery is referred to as day 0. In some mice, SNI surgery induced hypoesthesia/anesthesia in the territory of the paw innervated by the sural nerve, instead of inducing sensory hypersensitivity. This was considered to be a consequence of a failed surgery and the mice were discontinued from further evaluations. These mice accounted for less than 1% of the mice tested.

1.1.3. Drugs and drug administration

1.1.3.1. Acute treatment protocol

We used two selective Sig-1R ligands: the Sig-1 antagonist S1RA (E-52862.HCl; 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine) (8-128 mg/kg; DC Chemicals, Shanghai, China), and the Sig-1 agonist PRE-084 (2-[4-morpholinethyl]1-phenylcyclohexanecarboxylate hydrochloride) (Tocris Cookson Ltd., Bristol, United Kingdom) (Cobos et al., 2008). In addition, we used the following opioid receptor ligands: the opioid agonist morphine hydrochloride (0.5-2 mg/kg; General Directorate of Pharmacy and Drugs, Spanish Ministry of Health), the opioid antagonist naloxone hydrochloride and its peripherally restricted analog naloxone methiodide (Sigma-Aldrich, Madrid, Spain). The doses of S1RA and morphine used to reverse mechanical, heat and cold hypersensitivities were determined in the experiments shown in the "Results" section. The dose of PRE-084 used in the present study (32 mg/kg, s.c.) was selected based on our previous studies (Montilla-García et al., 2018; Entrena et al., 2009b). The doses of naloxone (1 mg/kg, s.c.) and naloxone methiodide (2 mg/kg, s.c.)

are also those used in our previous studies (Sánchez-Fernández et al., 2014a; Tejada et al., 2017). All drugs were dissolved to their final concentrations in sterile physiological saline just before administration, and were administered subcutaneously (s.c.) in the interscapular area in an injection volume of 5 mL/kg. The control animals received the same volume of the drug solvent (saline) s.c. All drugs were administered 7 days after surgery, when pain hypersensitivity was fully developed (see Fig 1), and their effects were tested as explained in section 1.1.4 below. When the effects of the association of two different drugs were evaluated, each injection was performed in a different area of the interscapular zone. In all cases, behavioral evaluations after drug administration were performed by an observer blinded to the treatment.

1.1.3.2. Repeated (10 days) treatment protocol

Treatment was given twice a day (every 12 hours) via the intraperitoneal (i.p.) route with S1RA 25 mg/kg or vehicle, since it has been previously described that S1RA was efficacious using this administration protocol in a model of neuropathic pain induced by nerve ligation (Romero et al., 2012). Treatment started in the day of surgery (first injection 30 min before the injury) and was maintained for up to day 9 (i.e. 10 days of treatment). The effects of treatments were evaluated on days 7 (30 min after the administration of S1RA or saline), 10 (12 hours after the last administration of S1RA or saline), 11 and 14 after nerve injury (36 and 108 hours after the last administration of S1RA or saline, respectively) in each animal. Behavioral evaluations after repeated drug administration were performed by an observer blinded to the treatment.

1.1.4. Behavioral assays

1.1.4.1. Time-course studies

To elucidate the time-course of SNI-induced pain hypersensitivity in WT and Sig-1R-KO mice, the behavioral responses were tested before surgery (baseline value). Then SNI surgery was performed and behavioral tests were carried out 3, 7, 14 and 21 days after SNI in each animal.

To investigate the acute effects of drugs on pain-related behaviors associated with SNI, presurgery baseline responses were evaluated, and then SNI surgery was performed. Seven days after the surgical procedure, when SNI-induced mechanical, heat and cold hypersensitivities were fully developed, pretreatment measurements were made (time 0) and then the drugs or saline were injected s.c., and the response of the animal to the nociceptive test was measured again 30, 90 and 180 min after the injection.

In all cases, each mouse was evaluated in only one nociceptive test and received drug treatment or saline only once. All behavioral evaluations were recorded by an observer blinded to the genotype and treatment.

1.1.4.2. Procedure to measure mechanical allodynia

Mechanical allodynia was assessed with von Frey filaments according to the up-down method (Chaplan et al., 1994), with slight modifications. On each day of evaluation the mice were habituated for 60 min in individual transparent plastic boxes (7 × 7 × 13 cm) placed on a wire mesh platforms. After the acclimation period, filaments were applied to the plantar ipsilateral hind paw in the sural nerve territory, pressed upward to cause a slight bend in the fiber, and left in place for 2-3 seconds. Calibrated von Frey monofilaments (Stoelting, Wood Dale, IL, USA) with bending forces that ranged from 0.02 to 1.4 g were applied using the up-down paradigm, starting with the 0.6 g filament and allowing 10 seconds between successive applications. The response to the filament was considered positive if immediate licking/biting, flinching or rapid withdrawal of the stimulated paw was observed. In each consecutive test, if there was no response to the filament, a stronger stimulus was then selected; if there was a positive response, a weaker one was then used.

1.1.4.3. Procedure to measure cold allodynia

Cold allodynia was tested by gently touching the plantar skin of the hind paw with an acetone drop, as previously described (Nieto et al., 2008). On each day of evaluation the mice were housed and habituated for 30 min in individual transparent plastic

enclosures (7 × 7 × 13 cm) with a floor made of wire mesh. Acetone was applied 3 times to the ipsilateral hind paw at intervals of 30 seconds, and the duration of biting or licking of the hind paw was recorded with a stopwatch and reported as the cumulative time of biting/licking in all 3 measurements. A cut-off time of 10 seconds was used in each of the three trials, because animals rarely licked their hind paw for more than 10 seconds. During the presurgery baseline evaluation we discarded ≈ 5 % of the mice tested due to an exaggerated atypical response to the acetone (> 5s of cumulative responses to acetone in the three measures).

1.1.4.4. Procedure to measure heat hyperalgesia

To measure heat hyperalgesia we used the Hargreaves method (Hargreaves et al., 1988), with slight modifications as previously described (Tejada et al., 2014). Mice were habituated for 2 hours in individual plastic chambers (9 × 9 × 22 cm) placed on a glass floor maintained at 30 °C. After habituation, a beam of radiant heat was focused to the plantar surface of the ipsilateral hind paw with a plantar test apparatus (IITC, Los Angeles, CA, USA), until the mouse made a withdrawal response. Each mouse was tested three times and the latencies were averaged for each animal. At least 60 seconds were allowed between consecutive measurements. A cut-off latency time of 20 seconds was used in each measurement to avoid lesions to the skin and unnecessary suffering.

1.1.5. Determination of the concentration of S1RA in plasma and brain tissue

Animals were treated as described in section 1.1.3 above, and the concentration of S1RA in plasma and brain tissue was measured 30 min and 12 hours after the last i.p. administration. Briefly, a terminal blood sample was drawn from each mouse by cardiac puncture at the appropriate time after vehicle or drug administration. Blood samples were collected in heparinized tubes and centrifuged at 2000 × g for 10 min to obtain plasma. Immediately after blood extraction, whole brains were removed. Plasma samples and brains were stored at −80 °C until analysis. Each brain was weighted and homogenized in 4 mL Dulbecco's phosphate buffered saline immediately

before drug concentrations were determined. Protein was precipitated with acetonitrile, and samples were analyzed by high-performance liquid chromatography-triple quadrupole mass spectrometry (HPLC-MS/MS) according to a previously described procedure (Romero et al., 2012). The concentration of the compound in plasma or brain was determined by least-squares linear regression with a calibration curve.

1.1.6. Data analysis

For behavioral studies, statistical analysis was carried out with two-way repeated measures analysis of variance (ANOVA). For the study of the S1RA levels determined by HPLC-MS/MS assay, statistical analysis was performed with two-way ANOVA. The Bonferroni post hoc test was performed in all cases. The differences between values were considered significant when the p-value was below 0.05. All data were analyzed with SigmaPlot 12.0 software (Systat Sofware Inc, San Jose, CA, USA).

1.2. Results

1.2.1. Comparison of SNI-induced neuropathic pain in sigma-1 receptor knockout and wild-type mice

We studied the involvement of the Sig-1R in neuropathic pain after SNI by comparing the response to mechanical, heat and cold stimuli in WT and Sig-1R-KO female mice. The baseline responses to the von Frey, Hargreaves and acetone tests before surgery did not differ significantly between Sig-1R-KO and WT animals in any group tested (Figs. 1A-C). In the sham-operated groups there were no significant postsurgery changes in the responses to any of the three behavioral tests in either Sig-1R-KO or WT animals (Figs. 1A-C). However, after SNI surgery, WT mice developed mechanical allodynia, manifested as a significant reduction in the mechanical threshold, which was detectable as early as day 3 after surgery, was greatest on day 7, and remained observable throughout the 21-day evaluation period. Sig-1R-KO mice also developed mechanical allodynia; however, it was significantly less pronounced than in WT mice, and the differences between WT and Sig-1R-KO mice were statistically significant from day 7 to day 21 (Fig. 1A). Both WT and Sig-1R-KO mice developed a similar degree of heat hypersensitivity in the Hargreaves test after SNI, with paw withdrawal latencies to heat stimulation significantly lower than those in sham-operated mice of both genotypes at all time points evaluated after SNI (Fig. 1B). Wild-type mice with SNI also developed marked cold allodynia, manifested as a significantly longer increase in the duration of paw licking/biting induced by acetone from day 3 throughout the evaluation period in comparison to the control WT sham group (Fig. 1C). In contrast, SNI surgery had no significant effect on the postsurgery responses in Sig-1R-KO mice in the acetone test, given that the values in this group were virtually identical to those in the Sig-1R-KO sham group throughout the evaluation period (Fig. 1C). These results are summarized in Table 1.

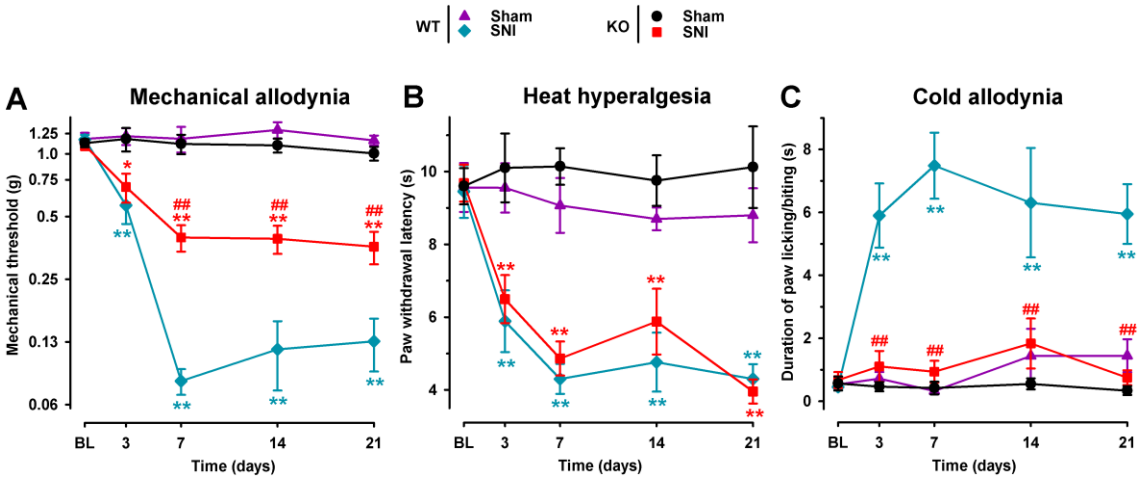

Figure 1. Comparison of spared nerve injury (SNI) -induced neuropathic pain behaviors in female wild-type (WT) and sigma-1 receptor knockout (Sig-1R-KO) mice. The von Frey threshold (A), latency to hind paw withdrawal in the Hargreaves test (B), and duration of hind paw licking or biting in the acetone test (C) were recorded 1 day before (baseline, BL) and on days 3, 7, 14 and 21 after surgery in the paw ipsilateral to the surgery. Each point and vertical line represent the mean ± SEM of the values obtained in 10-12 animals. Statistically significant differences between the values in the sham and SNI groups on the same day: *P < 0.05; **P < 0.01; and among WT and Sig-1R-KO groups: ##P < 0.01 (Two-way repeated measures ANOVA followed by Bonferroni test).

We also compared mechanical, heat and cold hypersensitivities induced by SNI in female and male animals from both genotypes. On day 7 after SNI, sensory hypersensitivity to the three types of stimuli was equivalent in WT female and male mice (Fig. 2). Sig-1R-KO mice of both sexes showed an equivalent reduction of mechanical allodynia, while showing the same extent of heat hyperalgesia than WT mice, but no cold allodynia (Fig. 2A, 2B and 2C, respectively).

In summary, SNI surgery induced mechanical, heat and cold hypersensitivity in WT mice of both sexes. However, SNI surgery led to a clearly different pattern of neuropathic signs in Sig-1R-KO mice irrespectively of the sex tested, as these female or male mutant mice developed heat hyperalgesia, but did not develop cold allodynia and showed significantly less mechanical allodynia.

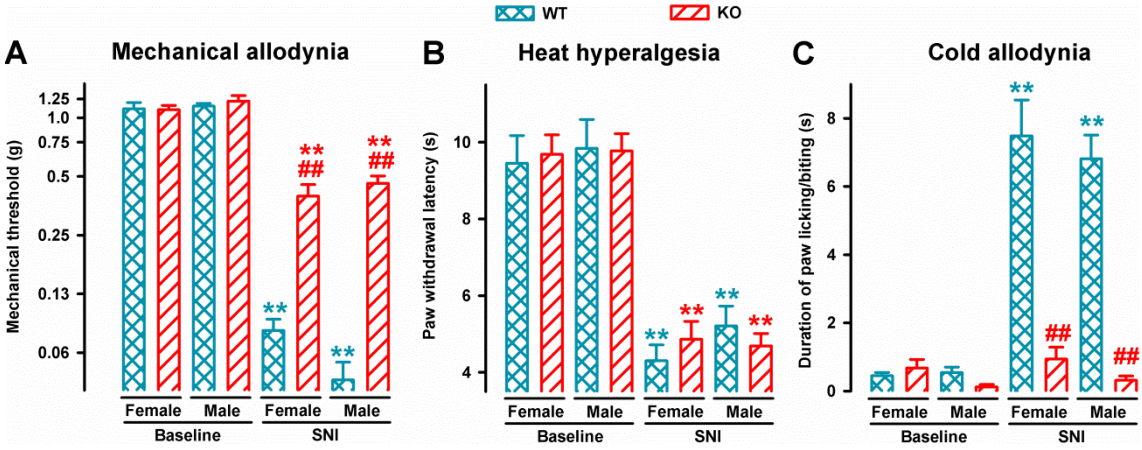

Figure 2. Comparison of spared nerve injury (SNI)-induced neuropathic pain behaviors in female and male wild-type (WT) and sigma-1 receptor knockout (Sig-1R-KO) mice. The von Frey threshold (A), latency to hind paw withdrawal in the Hargreaves test (B), and duration of hind paw licking or biting in the acetone test (C) were recorded 1 day before (baseline) and 7 days after surgery in the paw ipsilateral to the surgery. Each point and vertical line represent the mean ± SEM of the values obtained in 8-12 animals. Statistically significant differences between the values on the pre-surgery (baseline) day and 7 days after SNI in mice of the same sex: **P < 0.01; and between WT and Sig-1R-KO groups of mice of the same sex: ##P < 0.01. There were no statistical differences between values from mice of different sexes under the same experimental conditions (Two-way repeated measures ANOVA followed by Bonferroni test).

1.2.2. Effects of the acute systemic administration of the selective sigma-1 receptor antagonist S1RA on SNI-induced mechanical, cold and heat hypersensitivity

To test the effects of acute pharmacological antagonism by the Sig-1R on SNI-induced neuropathic pain, the selective Sig-1R antagonist S1RA was administered s.c. to female WT mice after neuropathy was fully developed (7 days after surgery). The threshold force needed to evoke pain-like responses before treatment with S1RA or saline was significantly lower than in the baseline measurement (Fig. 3A, time 0), thus showing mechanical allodynia. Saline administration did not significantly modify SNI-induced mechanical allodynia during the 3-hour test period (Fig. 3A). In contrast, the acute administration of S1RA (32-128 mg/kg) attenuated mechanical allodynia in a dose-dependent manner (Fig. 3A). In mice with SNI, paw withdrawal latencies to radiant heat were significantly shorter, in comparison to their baseline measurements, in all groups of animals before S1RA or saline administration (Fig. 3B, time 0). Saline administration did not significantly modify SNI-induced heat hyperalgesia, whereas the s.c. administration of S1RA (25-64 mg/kg) dose-dependently inhibited this response (Fig. 3B). In the acetone test, WT mice 7 days after SNI and before treatment with S1RA or saline (time 0) showed a longer duration of paw licking/biting in response to

acetone (Fig. 3C, time 0). In these mice, a single s.c. injection of saline did not modify the response to acetone at any of the time-points tested (Fig. 3C). However, a single s.c. injection of S1RA (8-64 mg/kg) dose-dependently reduced the duration of acetone-induced paw licking/biting from 30 to 90 min after treatment (Fig. 3C). Among the three sensory modalities explored in female mice, cold allodynia was the most sensitive outcome to the effects of S1RA, as it was fully reversed by 16 mg/kg of this compound, whereas S1RA 64 mg/kg was needed to fully reverse heat hyperalgesia, and we had to increase the dose of S1RA up to 128 mg/kg to induce a prominent (although partial) effect on SNI-induced mechanical allodynia (compare Figs. 3A-C). These results are summarized in Table 1.

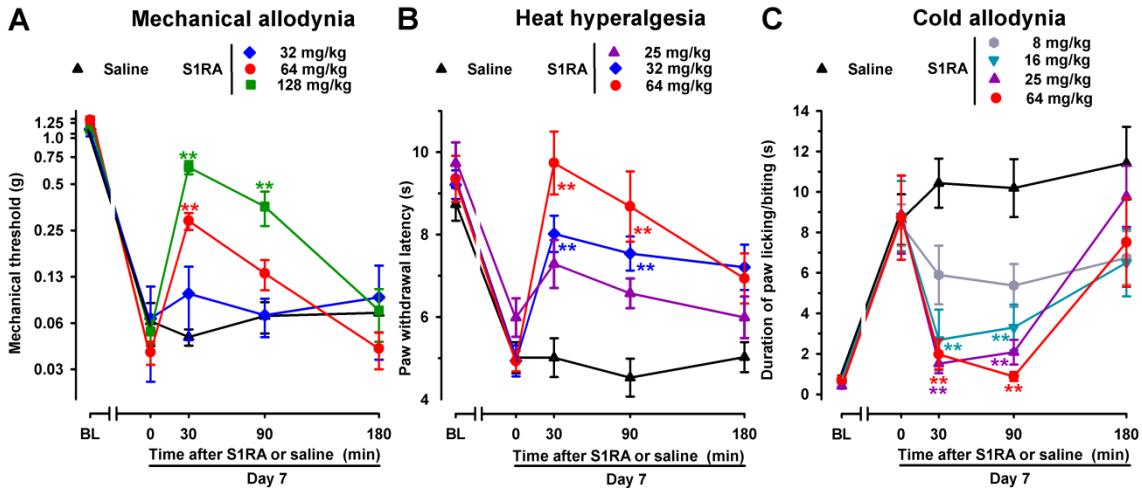

Figure 3. Time-course of the effects of a single s.c. injection of S1RA (8-128 mg/kg) or saline on mechanical allodynia (A), heat hyperalgesia (B) and cold allodynia (C), in female wild-type (WT) mice with SNI, 7 days after surgery. The von Frey threshold (A), the latency to hind paw withdrawal in the Hargreaves test (B), and the duration of hind paw licking or biting in the acetone test (C) were recorded 1 day before (baseline, BL) and 7 days (Day 7) after surgery in the paw ipsilateral to the surgery. On day 7, the responses to test stimuli was recorded immediately before (time 0) and at several times (30, 90 and 180 min) after injection of the drug or saline. Each point and vertical line represent the mean ± SEM of the values obtained in 8-11 animals. Statistically significant differences between S1RA- and saline-treated groups at the same time after treatment: **P < 0.01 (Two-way repeated measures ANOVA followed by Bonferroni test).

In contrast to the effect of the Sig-1R antagonist S1RA, the selective Sig-1R agonist PRE-084 (32 mg/kg, s.c.), when tested 7 days after SNI, did not alter SNI-induced mechanical-, heat- or cold-hypersensitivity in female WT mice (Figs. 4A, 4B and 4C, respectively). However, when PRE-084 (32 mg/kg, s.c.) and S1RA were associated, the antiallodynic and antihyperalgesic effects of this Sig-1R antagonist were abolished

(Figs. 4A-4C), suggesting that the effects of S1RA were mediated by the pharmacological antagonism of Sig-1R.

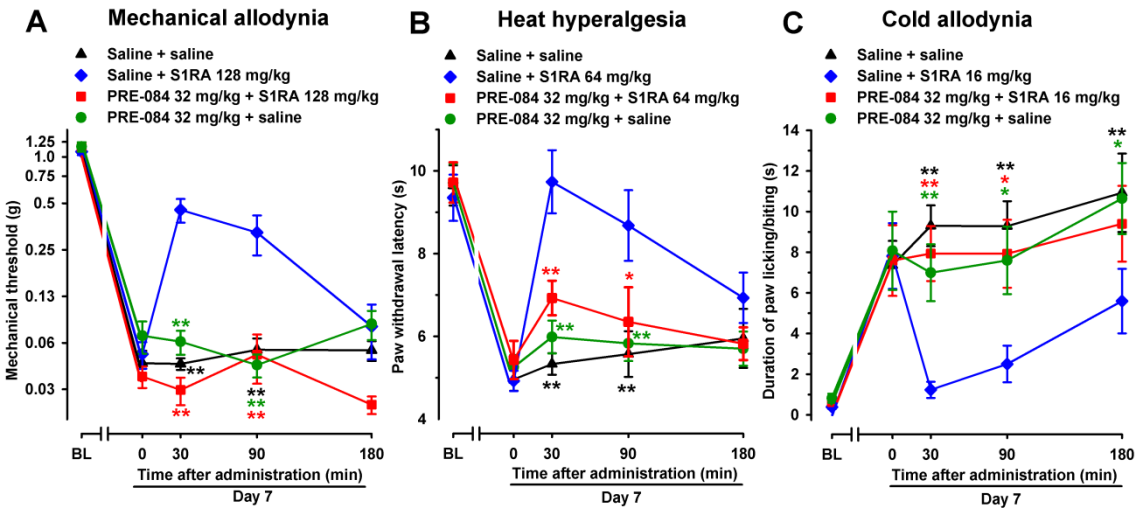

Figure 4. The Sig-1R agonist PRE-084 reversed the effects of the Sig-1R antagonist S1RA in female wild-type (WT) mice with SNI 7 days after surgery. Mechanical allodynia (A), heat hyperalgesia (B) and cold allodynia (C) were evaluated 1 day before (baseline, BL) and 7 days (Day 7) after surgery, in the paw ipsilateral to the surgery. On day 7, the responses to test stimuli were recorded immediately before (time 0) and at several times (30, 90 and 180 min) after injection of the drugs or saline. Each point and vertical line represent the mean ± SEM of the values obtained in 8-14 animals. Statistically significant differences in comparison to the saline+S1RA group: *P < 0.05; **P < 0.01 (Two-way repeated measures ANOVA followed by Bonferroni test).

To further verify the role Sig-1R on the effects induced by S1RA in SNI-induced hypersensitivity, we compared its effects in female WT and mice lacking Sig-1R (Sig-1R-KO mice). We tested the effects of this drug in Sig-1R-KO mice only for SNI-induced mechanical allodynia, which was partially developed in these mice, and heat hypersensitivity, which fully developed in Sig-1R-KO mice; whereas we did not test for the possible effects of S1RA on cold allodynia, since this type of hypersensitivity was absent in Sig-1R-KO mice (as shown in Fig. 1). WT mice given the Sig-1R antagonist S1RA showed less SNI-induced mechanical allodynia (Fig. 5A) and heat hyperalgesia (Fig. 5B), but the administration of S1RA to Sig-1R-KO mice did not induce significant antiallodynic or antihyperalgesic effects in these mutant mice (Fig. 5A and 5B).

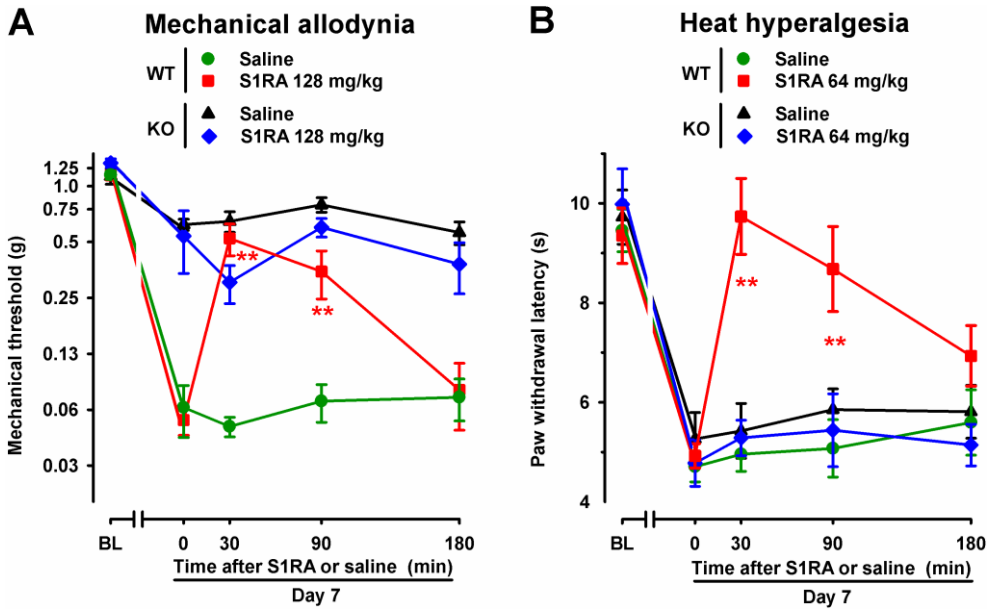

Figure 5. Time-course of the effects of a single s.c. injection of S1RA (64-128 mg/kg) or saline on mechanical allodynia (A) and heat hyperalgesia (B) in female wild-type (WT) and Sig-1R knockout (KO) mice with SNI 7 days after surgery. The von Frey threshold (A) and latency to hind paw withdrawal in the Hargreaves test (B) were evaluated 1 day before (baseline, BL) and 7 days (Day 7) after surgery in the paw ipsilateral to the surgery. On day 7 the responses to test stimuli were recorded immediately before (time 0) and at several times (30, 90 and 180 min) after injection of the drug or saline. Each point and vertical line represent the mean ± SEM of the values obtained in 8-14 animals. Statistically significant differences between S1RA- and saline-treated groups at the same time after treatment were found in WT mice (**P < 0.01) but not in Sig-1R-KO mice (Two-way repeated measures ANOVA followed by Bonferroni test).

Therefore, both the reversion of the effects of S1RA by PRE-084 and the absence of activity of S1RA in mice lacking Sig-1R suggest that off-target effects did not contribute to the antineuropathic effects of the Sig-1R antagonist S1RA in this neuropathic pain model.

1.2.3. Contribution of the endogenous opioid system to antineuropathic effects of the systemic administration of S1RA on SNI-induced neuropathic pain

In female WT mice, the association of the opioid antagonist naloxone (1 mg/kg, s.c.) with S1RA, administered 7 days after SNI surgery, completely reversed the ameliorative effects produced by the Sig-1R antagonist on hypersensitivity to mechanical (Fig. 6A) and heat stimuli (Fig. 6B). We also tested the effects of the peripherally restricted opioid antagonist naloxone methiodide on the antineuropathic effects of S1RA, and observed that peripheral opioid antagonism was also able to fully reverse the effects of S1RA on mechanical and heat hypersensitivity (Figs. 6A and 6B).

These data suggest that the effects induced by the Sig-1R antagonist on SNI-induced mechanical and heat hypersensitivity require the participation of the opioid system at the peripheral level. In contrast, naloxone treatment did not alter the effects of S1RA on cold allodynia (Fig. 6C), suggesting that opioid-independent effects induced by S1RA were involved in the decrease in this type of hypersensitivity. These results are summarized in Table 1.

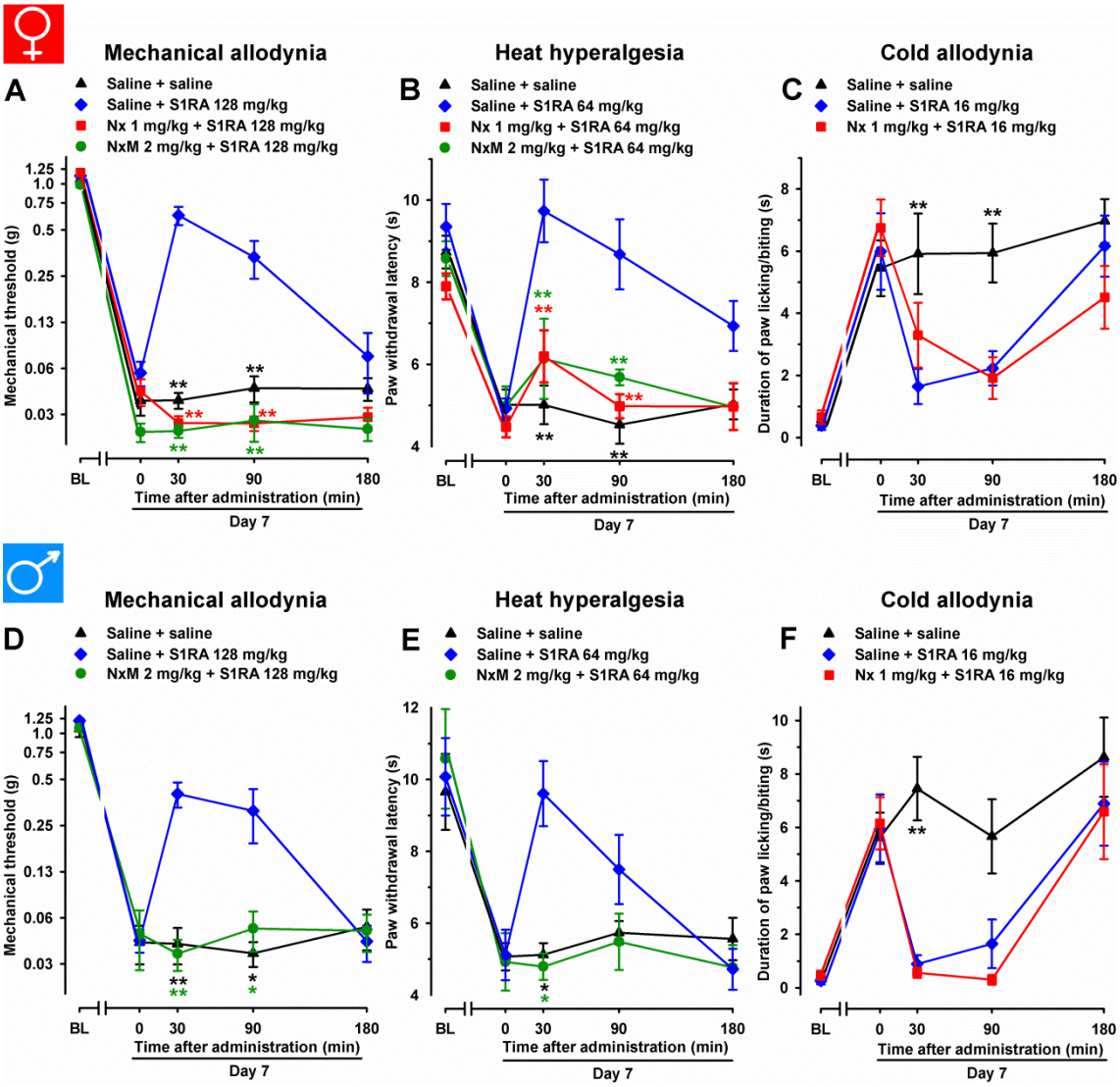

Figure 6. The opioid antagonists naloxone hydrochloride (Nx) and naloxone methiodide (NxM) reversed the effects of S1RA on mechanical allodynia and heat hyperalgesia but not on cold allodynia in female and male wild-type (WT) mice with SNI 7 days after surgery. In female mice, mechanical allodynia (A), heat hyperalgesia (B) and cold allodynia (C) were evaluated 1 day before (baseline, BL) and 7 days (Day 7) after surgery. Identical procedures were performed on male mice for the determination of mechanical (D) heat (E) and cold (F) sensitivity. On day 7 the responses to test stimuli in the paw ipsilateral to the surgery were recorded immediately before (time 0) and at several times (30, 90 and 180 min) after injections of the drugs (S1RA and opioid antagonists) or saline. Each point and vertical line represent the mean ± SEM of the values obtained in 8-14 animals. Statistically significant differences in comparison to the saline+S1RA group: **P < 0.01 (Two-way repeated measures ANOVA followed by Bonferroni test).

We tested these same doses of S1RA in male mice and found equivalent results than those found in female mice: the acute administration of the Sig-1R antagonist S1RA to male mice partially reversed SNI-induced mechanical allodynia but completely reversed heat- and cold- hypersensitivity (Fig. 6D, 6E and 6F, respectively). Similar to the results shown with female mice, the ameliorative effects of S1RA on mechanical and heat SNI-induced hypersensitivity in male mice were reversed by naloxone methiodide, whereas naloxone did not reverse the effects of S1RA on cold allodynia (Fig. 6D, 6E and 6F, respectively).

In order to explore whether the endogenous opioid system influences pain hypersensitivity induced by SNI in female mice, we administered naloxone (1 mg/kg) and naloxone methiodide (2 mg/kg) in the absence of S1RA to WT mice 7 days after SNI. No significant effects were observed with any of the opioid antagonists in any of the three sensory modalities explored (Figs. 7A-C).

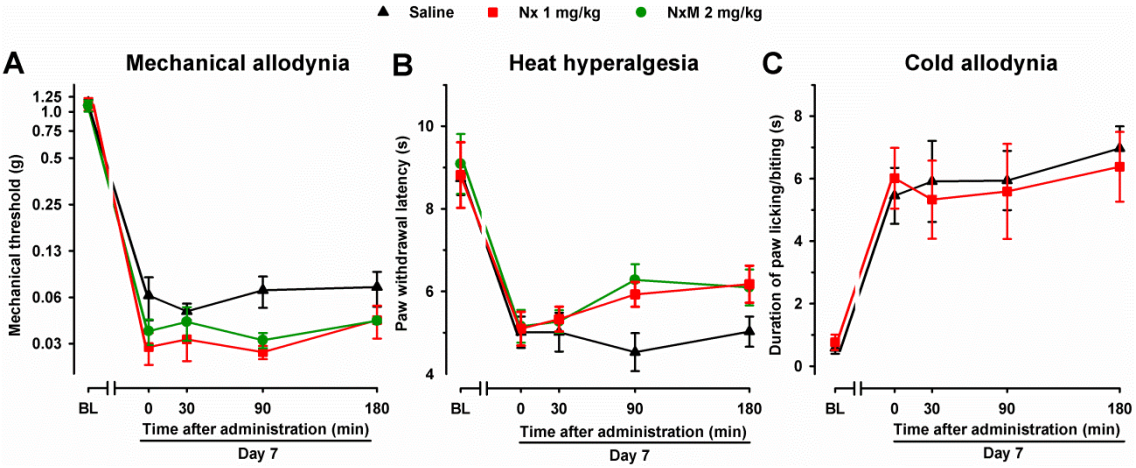

Figure 7. The opioid antagonists naloxone hydrochloride (Nx) and naloxone methiodide (NxM) had no effect *per se* in female wild-type (WT) mice with SNI 7 days after surgery. Mechanical allodynia (A), heat hyperalgesia (B) and cold allodynia (C) were evaluated 1 day before (baseline, BL) and 7 days (Day 7) after surgery in the paw ipsilateral to the surgery. On day 7 the responses to test stimuli were recorded immediately before (time 0) and at several times (30, 90 and 180 min) after injection of the opioid antagonist or saline. Each point and vertical line represent the mean ± SEM of the values obtained in 8-10 animals. Neither of the treatments produced statistically significant differences in comparison to the saline group (Two-way repeated measures ANOVA followed by Bonferroni test).

1.2.4. Effects of the systemic administration of morphine on SNI-induced cold and mechanical allodynia and heat hyperalgesia

To test the effects of an opioid drug on SNI-induced sensory hypersensitivity, we evaluated the effects of morphine in this neuropathic pain model in female mice. As expected, acute administration of the morphine solvent (saline) had no effect on hypersensitivity following SNI surgery (Figs. 8A-C). However, the administration of morphine (1 and 2 mg/kg, s.c.) led to significantly less mechanical allodynia associated with SNI, with a more prolonged effect at the highest dose tested (Fig. 8A). In addition, acute treatment with morphine (0.5 and 1 mg/kg) inhibited, in a dose-dependent manner, both heat hyperalgesia (Fig. 8B) and cold allodynia (Fig. 8C) induced by SNI. Whereas a single s.c. injection of morphine (1 mg/kg) completely reversed heat and cold hypersensitivity induced by SNI, the effect of morphine on SNI-induced mechanical allodynia was weaker, leading to only a partial reduction (see Figs. 8A-C).

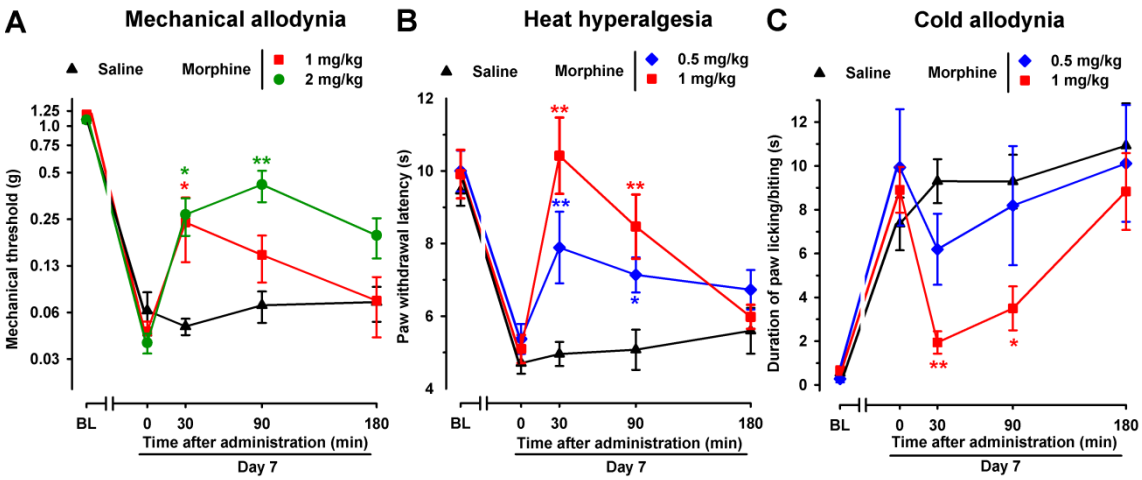

Figure 8. Time-course of the effects of a single s.c. injection of morphine (0.5-2 mg/kg) or saline on mechanical allodynia (A), heat hyperalgesia (B) and cold allodynia (C) in female wild-type (WT) mice with SNI 7 days after surgery. The von Frey threshold (A), latency to hind paw withdrawal in the Hargreaves test (B), and duration of hind paw licking or biting in the acetone test (C) were recorded 1 day before (baseline, BL) and 7 days (Day 7) after surgery in the paw ipsilateral to the surgery. On day 7 the responses to test stimuli were recorded immediately before (time 0) and at several times (30, 90 and 180 min) after injection of the drug or saline. Each point and vertical line represent the mean ± SEM of the values obtained in 8-13 animals. Statistically significant differences between morphine- and saline-treated groups at the same time after treatment: *P < 0.05; **P < 0.01 (Two-way repeated measures ANOVA followed by Bonferroni test).

To elucidate the possible contribution of peripheral opioid receptors to the antinociceptive effects induced by morphine in SNI mice, we associated morphine administration with the injection of the opioid antagonists naloxone or naloxone

methiodide. As expected, naloxone (1 mg/kg s.c.) completely reversed the antinociceptive effect of morphine in all three sensory modalities explored, with values indistinguishable from those in mice treated with the drug solvent (Figs. 9A-C). However, naloxone methiodide (2 mg/kg) completely reversed the effect of morphine (1 mg/kg) on mechanical allodynia (Fig. 9A), and markedly reduced its effects on heat hyperalgesia (Fig. 9B), whereas it did not reverse the effect of morphine on SNI-induced cold allodynia (Fig. 9C). These data suggest that peripheral opioid receptors contributed to the ameliorative effects induced by morphine only in hypersensitivity to mechanical and heat stimuli induced by SNI, but not in cold allodynia.

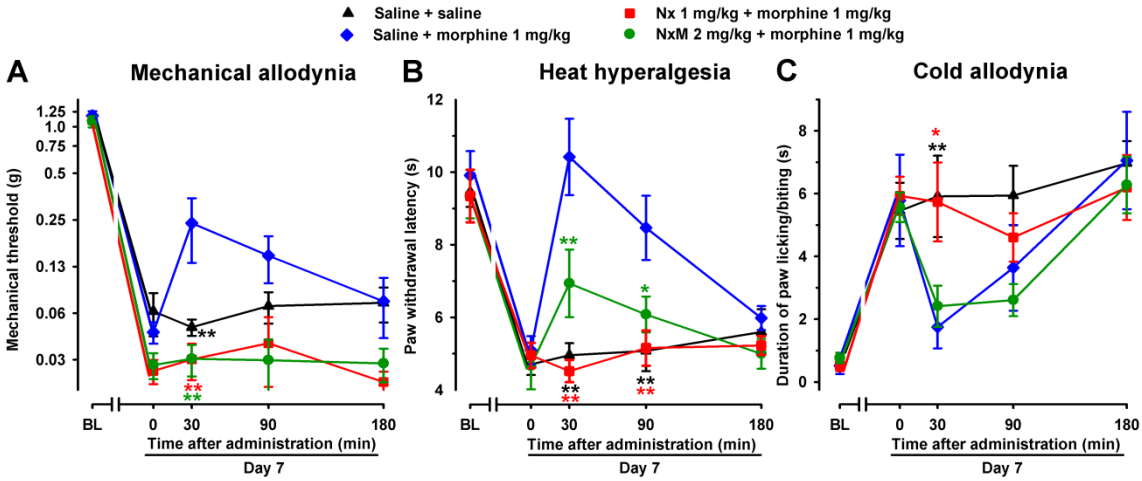

Figure 9. Differential ability of naloxone hydrochloride (Nx) and naloxone methiodide (NxM) to reverse the effects of morphine on mechanical allodynia, heat hyperalgesia and cold allodynia in female wild-type (WT) mice with SNI 7 days after surgery. Mechanical allodynia (A), heat hyperalgesia (B) and cold allodynia (C) were evaluated 1 day before (baseline, BL) and 7 days (Day 7) after surgery in the paw ipsilateral to the surgery. On day 7 the responses to test stimuli were recorded immediately before (time 0) and at several times (30, 90 and 180 min) after injections of the drugs (morphine and opioid antagonists) or saline. Each point and vertical line represent the mean ± SEM of the values obtained in 8-14 animals. Statistically significant differences in comparison to the saline+morphine group: *P < 0.05; **P < 0.01 (Two-way repeated measures ANOVA followed by Bonferroni test).

1.2.5. Effect of repeated treatment with S1RA on neuropathic pain-related behaviors

To study the effect of prolonged pharmacological antagonism of the Sig-1R on the development of SNI-induced neuropathy, we administered to WT female mice two daily injections of S1RA (25 mg/kg, i.p.) or saline, starting 30 min before surgery and continuing up to day 9. Mechanical allodynia (Fig. 10A), heat hyperalgesia (Fig. 10B) and cold allodynia (Fig. 10C) induced by SNI were suppressed by the repeated

administration of S1RA when measured on day 7 after surgery, 30 min after its administration.

The antineuropathic effects induced by repeated treatment with S1RA were still significant in all three sensory modalities explored (compared to treatment with the vehicle only) on day 10, 12 hours after the last administration of S1RA (Figs. 10A-10C). However, the antineuropathic effects of S1RA disappeared in longer periods after treatment was discontinued: allodynia and hyperalgesia values on days 11 and 14 were indistinguishable from those in the vehicle-treated group (Figs. 10A-10C).

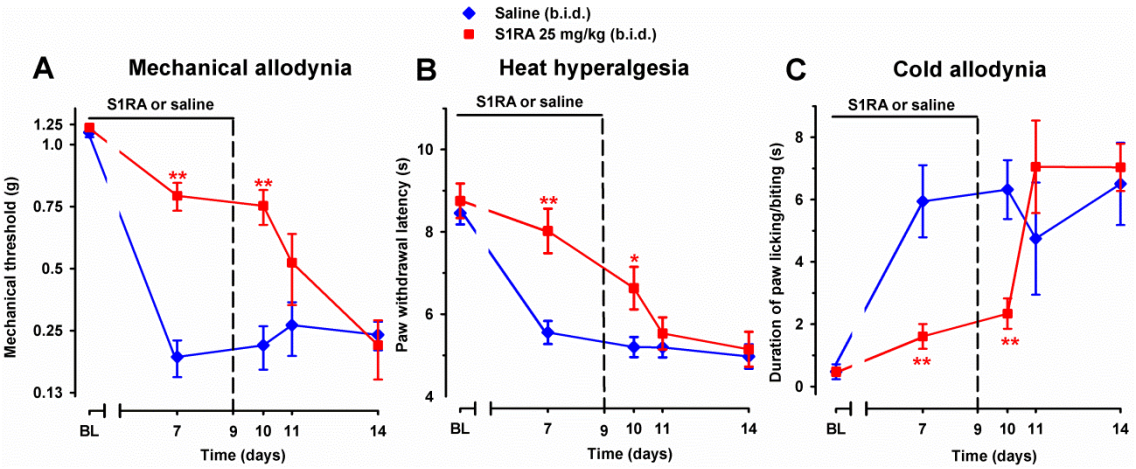

Figure 10. Time-course of the effect of repeated treatment with S1RA on mechanical allodynia (A), heat hyperalgesia (B) and cold allodynia (C) induced by SNI in female wild-type (WT) mice. The mice were treated twice daily with either saline or S1RA (25 mg/kg, i.p.). The first injection was administrated 30 min before SNI surgery. Responses were recorded in each animal before SNI (baseline, BL) and 30 min after the administration of S1RA or saline on day 7 in the paw ipsilateral to the surgery. Treatment was continued until day 9 and the mice were evaluated again on days 10, 11 and 14 postsurgery. The black horizontal line at the top of each figure illustrates the duration of treatment with S1RA or saline. Each point and vertical line represent the mean ± SEM of the values obtained in 10-14 animals. Statistically significant differences between S1RA- and saline-treated groups on the same day after treatment: *P < 0.05; **P < 0.01 (Two-way repeated measures ANOVA followed by Bonferroni test).

In contrast, acute treatment with S1RA (25 mg/kg, i.p.) had no significant effect on mechanical or heat hypersensitivities (Figs. 11A and 11B), but significantly inhibited SNI-induced cold hypersensitivity (Fig. 11C), in agreement with the previously commented higher sensitivity of S1RA effects on SNI-induced cold allodynia with respect to mechanical and heat hypersensitivity. The effects of this dose of S1RA lasted longer when administered s.c. than i.p. (compare Fig. 3C and 11C), suggesting a faster drug elimination of the later.

Taking into account that the acute administration of a dose of S1RA which lacks of effect on mechanical and heat hypersensitivity and induced only a transient effect on

cold allodynia, but the repeated treatment with this same dose of S1RA induced a marked and long-lasting effect on the three outcomes examined, we conclude that repeated treatment with this drug results in an improvement of its effects.

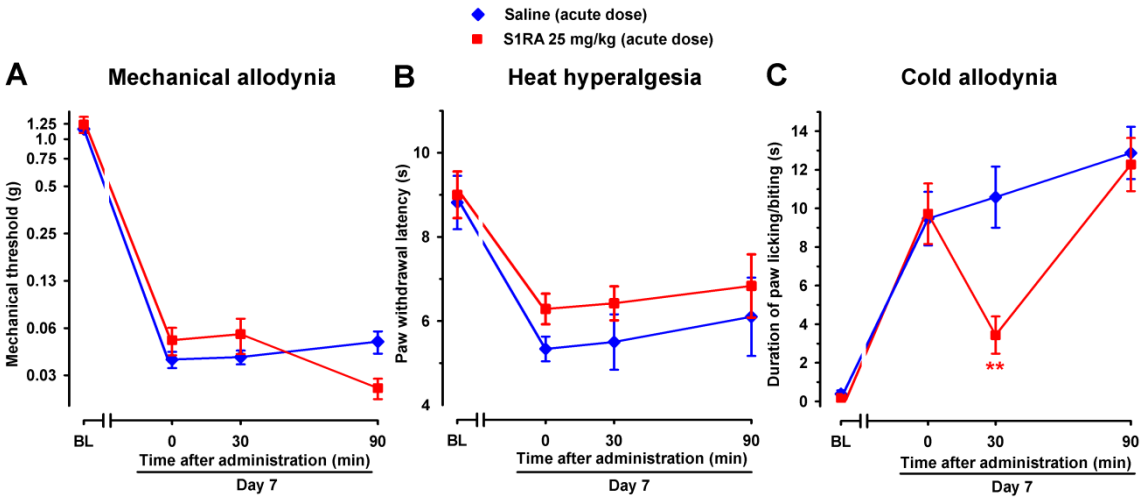

Figure 11. Time-course of the effects of a single i.p. injection of S1RA (25 mg/kg) or saline on mechanical allodynia (A), heat hyperalgesia (B) and cold allodynia (C) in female wild-type (WT) mice with SNI 7 days after surgery. Responses were recorded in each animal before SNI (baseline, BL) and 7 days (Day 7) after surgery. On day 7 the responses to test stimuli in the paw ipsilateral to the surgery were recorded immediately before (time 0) and at two times (30 and 90 min) after injection of the drug or saline. Each point and vertical line represent the mean ± SEM of the values obtained in 6-8 animals. Statistically significant differences between S1RA- and saline-treated groups at the same time after treatment: **P < 0.01 (Two-way repeated measures ANOVA followed by Bonferroni test).

1.2.6. Concentration of S1RA in plasma and brain tissue after repeated administration

To test whether the sustained antinociceptive effects induced by the repeated administration of S1RA 12 hours after the discontinuation of drug treatment (i.e. on day 10) was due to the presence of any drug remaining in the organism, the concentrations of S1RA in plasma and brain tissue were measured 30 min and 12 hours after the last dose of S1RA. On day 9 of repeated treatment, 30 min after the last administration of S1RA (25 mg/kg, i.p.), we found significant levels of this Sig-1R antagonist in both plasma and brain, with a much higher concentration in the latter (red bars in Figs. 12A and 12B). In contrast, 12 hours after the last administration, we observed no appreciable levels of this Sig-1R antagonist in any sample analyzed (Figs. 12A and 12B).

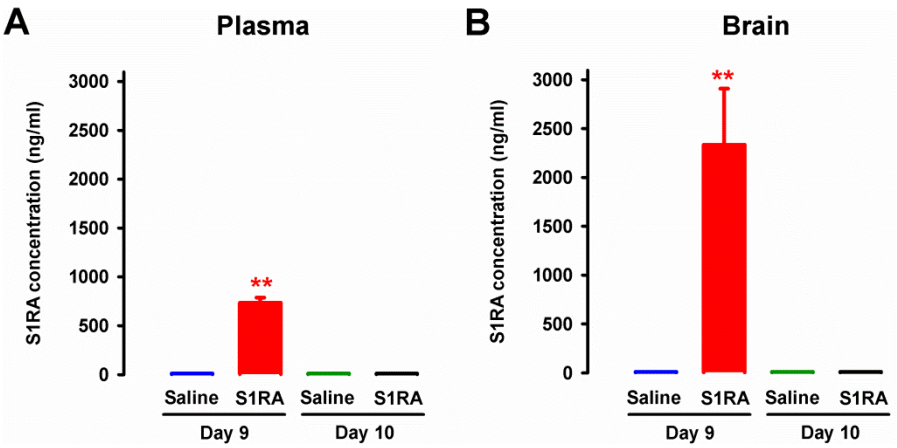

Figure 12. Concentrations of S1RA in plasma and brain tissue after its repeated administration in female wild-type (WT) mice. The levels of S1RA were measured by high-performance liquid chromatography-triple quadrupole mass spectrometry (HPLC-MS/MS) in plasma (A) and brain homogenates (B). Mice were treated from day 0 to day 9 twice daily with either saline or S1RA (25 mg/kg, i.p.). Plasma and brain samples were obtained on day 9 (30 min after S1RA or saline administration) and day 10 (12 h after S1RA or saline administration). Each bar and vertical line represent the mean ± SEM of the values obtained in 5-6 animals (A and B). Statistically significant differences between the levels 30 min after S1RA administration and the rest of the experimental groups: **P < 0.01 (Two-way ANOVA followed by Bonferroni test).

Table 1. Summary of the main results obtained in this study on the effect of the acute administration of S1RA to female wild-type mice and the effects seen on of sigma-1 receptor knockout (Sig-1R-KO) female mice on the Spared Nerve Injury model of neuropathic pain. The figures that show the results for the different experiments are indicated.

Type of sensory hypersensitivity	Wild-type mice									Sig-1R-KO mice			
	Sensitivity to S1RA			Reversion of the S1RA antineuropathic effect						Without any treatment *		S1RA effects	
	Efficacy *	Potency *		PRE-084		Naloxone *		Naloxone methiodide *					
Mechanical allodynia	++	+	Fig 3A	Yes	Fig 4A	Yes	Fig 6A	Yes	Fig. 6A	Partially reduced	Fig 1A	Absent	Fig 5A
Heat hyperalgesia	+++	++	Fig 3B	Yes	Fig 4B	Yes	Fig 6B	Yes	Fig. 6B	Fully present	Fig 1B	Absent	Fig 5B
Cold allodynia	+++	+++	Fig 3C	Yes	Fig 4C	No	Fig 6C	Not tested		Absent	Fig 1C	Not tested	

*Experiments where male mice were also tested with similar results to female mice (see Figs. 2 and 6).

1.3. Discussion

The main findings of the present study are that: 1) pharmacological antagonism or genetic inactivation of Sig-1R reduces neuropathic pain induced by peripheral nerve transection (SNI model); 2) the ameliorative effects on SNI-induced hypersensitivity to mechanical and heat stimuli (but not to cold stimuli) produced by Sig-1R antagonism are mediated by modulation of the endogenous opioid system; and 3) repeated treatment with S1RA induces prolonged ameliorative effects which lasted longer than the presence of the drug in the organism.

Basal sensitivity to mechanical, cold and heat stimulation in mice lacking Sig-1R, in the absence of nerve injury, did not differ from that in WT mice. This is in agreement with previous studies (de la Puente et al., 2009; Nieto et al., 2012) and suggests that the basic mechanisms of nociceptive transduction are intact in mice lacking Sig-1R. We showed that WT mice after SNI surgery developed mechanical, cold and heat hypersensitivities with time courses similar to those previously reported in mice (Bourquin et al., 2006; Tejada et al., 2014; Guida et al., 2015; Liu et al., 2015; Cobos et al., 2018). In addition, the extent of the sensory hypersensitivity was similar between female and male WT mice. In mice lacking Sig-1R from either sex, however, SNI surgery induced a very different pattern of painful hypersensitivity: these mutant mice did not develop cold allodynia and showed significantly less mechanical allodynia, whereas they developed heat hyperalgesia normally. This is consistent with previous work in other neuropathic pain models, which found that Sig-1R-KO mice had significantly reduced mechanical and cold allodynia induced by chemotherapy (Nieto et al., 2012) or by partial sciatic nerve ligation (de la Puente et al., 2009); the latter study also found that heat hyperalgesia developed normally (de la Puente et al., 2009). However, it was recently reported that Sig-1R-KO mice showed, in addition to the reduction in mechanical allodynia, a significant attenuation of heat hypersensitivity induced by spinal cord injury (Castany et al., 2018) or diabetic neuropathy (Wang et al., 2018). Taken together, studies with Sig-1R-KO mice suggest that the role of Sig-1R during neuropathic pain depends on the sensory modality explored and the type of injury.

The acute pharmacological antagonism of Sig-1R with S1RA administration, once the painful neuropathy was fully established in WT mice, significantly (although partially) attenuated mechanical allodynia and fully reversed cold hypersensitivity induced by SNI. In addition, and in contrast to our findings with Sig-1R-KO mice, acute S1RA administration also abolished SNI-induced heat hyperalgesia in female or male WT mice. Previous studies in other neuropathic pain models reported that the systemic acute administration of Sig-1R antagonists (including S1RA) reversed not only neuropathic cold and mechanical allodynia (Nieto et al., 2012; Romero et al., 2012; Gris et al., 2016; Espinosa-Juárez et al., 2017a; Paniagua et al., 2017; Castany et al., 2018; Wang et al., 2018) but also heat hyperalgesia (Díaz et al., 2012; Romero et al., 2012; Paniagua et al., 2017; Castany et al., 2018; Wang et al., 2018), findings which are also in clear agreement with our results. Therefore, there is an apparent divergence between the reduction in heat hyperalgesia in S1RA-treated WT mice, and the normal development of heat hypersensitivity in Sig-1R-KO mice after SNI. This inconsistency cannot be explained by a nonspecific off-target effect of S1RA, because here we show that the acute ameliorative effects induced by S1RA in all three sensory modalities explored in the present study were abolished by the selective Sig-1R agonist PRE-084, which did not show any effects "per se", as previously described in a different neuropathic pain model (Espinosa-Juárez et al., 2017a). In addition, the administration of S1RA to Sig-1R-KO mice had no effect on SNI-induced mechanical and heat hypersensitivity. Therefore, these results argue in favor of a Sig-1R-mediated action induced by this drug. In fact, it is known that S1RA has exquisite selectivity for Sig-1R (Romero et al., 2012). The discrepancy between the effect of genetic and pharmacological inhibition of Sig-1R on heat hypersensitivity was also found in other studies of neuropathic pain (de la Puente et al., 2009; Romero et al., 2012) and during inflammatory pain (Tejada et al., 2014). In addition, conflicting results between sigma-1 knockout and pharmacological antagonism have been reported in the modulation of opioid-induced analgesia in nociceptive heat pain (Vidal-Torres et al., 2013). Studies of pain mechanisms that used the genetic and pharmacological inhibition of other receptors have also obtained contradictory results (Petrus et al., 2007; Bonin et al., 2011), which have been attributed to compensatory mechanisms developed by mutant mice. Therefore, this issue appears to be a general concern in experiments with

knockout animals. Taking into account these antecedents, we suggest that Sig-1R-KO mice develop these purported compensatory mechanisms in heat pain pathways but not in other pain pathways in which the knockout replicated the effects of Sig-1R antagonists.

After peripheral nerve injury, neuroinflammatory processes occur with the recruitment of myriad immune cells at the site of injury (Treutlein et al., 2018) and in the dorsal root ganglia (Kwon et al., 2013), where the somas of peripheral sensory neurons are located. These immune cells release a variety of inflammatory mediators that contribute to neuropathic pain, but they also produce EOPs which have analgesic potential (reviewed in Ji et al., 2014; Stein, 2016; Tejada et al., 2018). In our experimental conditions, opioid antagonism caused by the administration of naloxone or its peripherally restricted analog naloxone methiodide did not exacerbate pain hypersensitivity in any sensory modality explored, suggesting that the tonic endogenous activity of the opioid system is limited in SNI mice. Importantly, the ameliorative effects induced by S1RA in SNI-induced mechanical and heat hypersensitivity were reversed by both naloxone and naloxone methiodide. These results suggest that Sig-1R inhibition ameliorates SNI-induced mechanical and heat hypersensitivity through a mechanism dependent on peripherally-produced EOPs, whose actions are tonically limited by Sig-1R. This dependence on the peripheral opioid system of the effects induced by S1RA on mechanical and heat hypersensitivity was seen in both female and male mice, indicating a lack of sexual dimorphism in these effects. It was recently reported that Sig-1R antagonism produced opioid-dependent antihyperalgesic effects during inflammation by enhancing the action of EOPs released by immune cells that accumulate at the inflamed site (Tejada et al., 2017). Interestingly, Sig-1R are expressed in the somas of all peripheral sensory neurons (Mavlyutov et al., 2016; Montilla-García et al., 2018), at a much higher density than in central pain-related areas (Sánchez-Fernández et al., 2014a). In light of these antecedents, it can be hypothesized that our results for the effects of S1RA on neuropathic mechanical and heat hypersensitivity may also be attributable to the enhancement, by Sig-1R antagonism, of the peripheral antinociceptive actions of EOPs produced by immune cells. Further research is guaranteed to determine the exact

EOP/EOPs involved in the opioid-dependent effects induced by Sig-1R antagonism during neuropathic pain.

It has been reported that peripheral immune cells do not contribute equally to every modality of sensory hypersensitivity after peripheral nerve injury. In fact, peripheral macrophages and T-cells promote both mechanical allodynia and heat hyperalgesia (Kobayashi et al., 2015; Cobos et al., 2018), whereas their influence in cold allodynia is very limited, which suggest that it is due to neuronal mechanisms rather than to neuro-immune interactions (Cobos et al., 2018). Here we show that the effects of S1RA on cold allodynia in either female or male mice are independent of opioid activation, which is consistent with the known inhibitory effects of S1RA on neuronal sensitization (Romero et al., 2012; Paniagua et al., 2017). In this connection it was previously reported that the effects of Sig-1R antagonists in other pain models such as capsaicin-induced secondary hypersensitivity (Entrena et al., 2009b) or formalin-induced pain (Tejada et al., 2017) were not sensitive to naloxone treatment. Taking into account the wide variety of protein partners (other than opioid receptors) that benefit from the chaperoning actions of Sig-1R (reviewed in Su et al., 2016; Sánchez-Fernández et al., 2017), it is not surprising that multiple opioid and non-opioid mechanisms simultaneously participate on the ameliorative effects of Sig-1R antagonism.

We also found that whereas morphine only partially reversed mechanical allodynia, it was able to fully suppress heat and cold hypersensitivity induced by SNI; effects which resemble those induced by S1RA. The effects of morphine on mechanical and heat hypersensitivity, but not on cold allodynia, were sensitive to the peripheral opioid antagonist naloxone methiodide. These results suggest that peripheral opioid effects are markedly weaker in cold allodynia than in mechanical or heat hypersensitivity, and are consistent with the absence of peripherally-mediated opioid effects on the inhibition of cold allodynia induced by S1RA.

We also tested the effects of the repeated administration of S1RA, which was administered preemptively before surgery and subsequently administered twice a day during the next 9 days. Plasma levels of S1RA in mice after repeated treatment with this drug were similar to or lower than the levels of this drug in humans after daily oral

S1RA treatment at therapeutic doses (Abadias et al., 2013; Bruna et al., 2018). We found that the sustained administration of S1RA induced prolonged ameliorative effects on the hypersensitivity to mechanical, heat and cold stimuli, without any evidence of tolerance to the antineuropathic effects during the evaluation period. This may be relevant given that some effects of S1RA, as shown in the present study, involve the opioid system, and it is well known that sustained opioid treatment induced analgesic tolerance (Morgan and Christie, 2011).Therefore, although we show that the effects of Sig-1R antagonism are partially mediated by the opioid system, this does not necessarily imply the development of analgesic tolerance.

We evaluated the effects of the repeated administration of S1RA starting before neuropathic pain was established, and found that it had marked effects on mechanical, heat and cold hypersensitivity. However, when the same dose of S1RA was acutely administered once neuropathic pain was fully established, its effects were limited and observed only in cold allodynia. Therefore, S1RA showed higher efficacy after repeated treatment than after a single treatment. It is unlikely that the greater effects induced by repeated treatment of S1RA were due to drug accumulation, since we previously showed that repeated treatment with the same protocol as in the present study did not result in increased levels of S1RA with time (Romero et al., 2012). In addition, here we show that 12 hours after treatment was discontinued, there were no appreciable levels of S1RA in either plasma or brain tissue, indicating the complete elimination of this compound between doses. Interestingly, although no S1RA remained in the organism 12 hours after its last administration, drug effects were still significantly evident in all three sensory modalities. Our results are consistent with previous studies in which the repeated administration of Sig-1R antagonists (including S1RA) consistently induced a long-lasting reduction of the development of mechanical, cold and heat hypersensitivity in models of neuropathic pain of different etiologies (Nieto et al., 2012; Gris et al., 2016; Paniagua et al., 2017). It is unclear whether these prolonged effects induced by the repeated treatment with S1RA might be due to the production of an active metabolite not detected in our determinations. However, it is known that Sig-1R can influence gene transcription, which might account for the long-lasting effects observed (Tsai et al., 2015). In fact, repeated treatment with the Sig-1R

agonist PRE-084, which is chemically unrelated to S1RA, had long-lasting proallodynic effects (Entrena et al., 2016). Taken together, these results suggest that the repeated treatment with Sig-1R ligands might have sustained pharmacodynamic effects, not restricted to S1RA and its possible active metabolites, although further experiments are needed to clarify this issue. Regardless of the precise mechanism, our data suggest that repeated treatment with S1RA may have potential therapeutic utility in the context of neuropathies induced by nerve transection during surgery, when the precise time of nerve injury can be anticipated and preventive treatment can be given.

In summary, this study demonstrates that Sig-1R antagonism may be a potentially effective therapeutic tool to inhibit neuropathic pain induced by peripheral nerve transection. In addition, our findings support the notion that Sig-1R antagonism induces both opioid-dependent and -independent effects during neuropathic pain.

2. STUDY 2: Sigma-1 receptors control neuropathic pain and macrophage infiltration into the dorsal root ganglion after peripheral nerve injury

2.1. Material and methods

2.1.1. Animals

Experiments were performed in female WT (Charles River, Barcelona, Spain) and Sig-1R-KO CD-1 mice (Laboratorios Esteve, Barcelona, Spain) weighing 26-32 g. The animals were housed in colony cages with free access to food and water prior to the experiments. They were maintained in light- and temperature -controlled rooms (23 ± 2 °C, lights on at 07.00 h and off at 19.00 h). The animals were tested at random times throughout the estrous cycle. Animal care was in accordance with institutional (Research Ethics Committee of the University of Granada, Spain), regional (Junta de Andalucía, Spain) and international standards (European Communities Council Directive 2010/63). To decrease the number of animals in this study, we used the same mice for behavioral and in vitro studies, when possible.

2.1.2. Spared nerve injury

The spared nerve injury (SNI) was induced as previously described (32). Briefly, an incision was made in the left skin at the site of trifurcation of the sciatic nerve, and its three terminal branches (the sural, common peroneal and tibial nerves) were exposed. The tibial and common peroneal branches were ligated with a silk suture and transected distally, while the sural nerve was left intact. In sham-operated control mice, the sciatic nerve terminal branches were exposed but not ligated. Mice were anesthetized with isoflurane 3% (Braun VetCare, Barcelona, Spain) during the procedure.

2.1.3. Assessment of mechanical allodynia

Mechanical thresholds were tested before surgery (baseline) and 3, 7 and 14 days after SNI. Mechanical allodynia was assessed with von Frey filaments according to a

previously described method, with slight modifications (Chaplan et al., 1994). On each day of evaluation the mice were habituated for 60 min in individual transparent plastic chambers (7 × 7 × 13 cm) with a floor made of wire mesh. After the adaptation period, calibrated von Frey monofilaments (Stoelting, Wood Dale, IL, USA) with bending forces that ranged from 0.02 to 2 g were applied with the up-down paradigm in the sural nerve territory, starting with the 0.6 g filament. The response to the filament was considered positive if immediate flinching, licking/biting or rapid withdrawal of the stimulated paw was observed. In each consecutive test, if there was a positive response, a weaker filament was then used; if there was no response to the filament, a stronger stimulus was then selected. Behavioral evaluations were performed by an observer blinded to the mouse genotypes in all experiements.

2.1.4. Immunohistochemistry

All experiments were performed in lumbar L4 DRG, since the somas of the common peroneal/tibial branches of the sciatic nerve and those from the sural nerve in mice are located at this level (Laedermann et al., 2014). This made it possible to immunostain injured and non-injured neurons at this location simultaneously.

On day 7 after surgery, SNI mice were anesthetized with isoflurane 4% and perfused transcardially with 0.9% saline solution followed by 4% paraformaldehyde (Sigma-Aldrich, Madrid, Spain). The DRG were excised and post-fixed for 1 h (4 °C) in the same fixative solution. Samples were dehydrated and embedded in paraffin with standard produces. Sections of DRG were serially cut on a sliding microtome at 5 µm and mounted on microscope slides (Sigma-Aldrich). Slides containing every tenth section of L4 DRG were deparaffinized in xylol (Panreac Quimica, Castellar del Vàlles, Spain), and rehydrated with alcohol (Panreac Quimica) and distilled water before the antigen retrieval procedure (steam heating for 22 min with 1% citrate buffer, pH 8). Tissue sections were incubated for 60 min in a blocking solution that contained 5% normal goat or horse serum (Sigma-Aldrich) depending on the experiment, 0.3% Triton X-100 (Sigma-Aldrich) and 0.1% Tween 20 (Sigma-Aldrich) in Tris buffer solution at room temperature (RT). Then the slides were incubated with the primary antibodies in

blocking solution. The primary antibodies used were rabbit anti-ATF3 (1:200, sc-188, Santa Cruz Biotechnology, Inc., Heidelberg, Germany), mouse anti-Sig-1R (1:200, sc-137075, Santa Cruz Biotechnology), rabbit anti-ionized calcium-binding adapter molecule 1 (IBA-1, 1:200, 019-19741, Wako Chemical, Neuss, Germany), or rabbit anti-NeuN (neuronal nuclei) (1:500, ABN78, Merck Millipore, MA, USA). Incubation with the primary antibodies for Sig-1R, NeuN and IBA-1 lasted for 1 h (RT), whereas incubation with the primary antibody for ATF3 lasted overnight (4 °C). After incubation with the primary antibodies, the tissue sections were washed three times for 10 min and incubated for 1 h (RT) with the appropriate secondary antibody solution containing goat anti-mouse Alexa Fluor 488 (A11029), goat anti-rabbit Alexa Fluor 488 (A11008), or goat anti-rabbit Alexa Fluor 594 (A11012) (all 1:500, all from Life Technologies, Carlsbad, CA, USA), depending on the experiment. We also stained some tissue sections with NeuN conjugated with Alexa Fluor 555 (1:500, MAB377A5, Merck Millipore).

The slides were then washed three times for 10 min and coverslipped with ProLong® Gold Antifade mounting medium (Molecular Probes; Oregon, USA). In some experiments, slides were incubated for 5 min (RT) with Hoescht 33342 (1:1000, Sigma-Aldrich) and washed three times before the mounting procedure. Slides were visualized under a confocal laser-scanning microscope (Model A1, Nikon Instruments Europe BV, Amsterdam, Netherlands) and processed with Image-J software (version 1.48, Wayne Rasband, NIH, Bethesda, MD, USA). For quantifications based on inmunohistochemical images, the data were obtained in 3-6 ipsilateral or contralateral sections per mouse, from at least 4 mice. To study macrophage infiltration into the DRG, double positive immunoreactivity for IBA-1 (as a macrophage/monocyte marker) and Hoechst 33342 was quantified in 4 areas (100 × 100 μm) located randomly in each DRG section. For the remaining quantifications, whole DRG sections were used instead of random areas. To estimate the percentage of DRG neurons with macrophage ring-like structures closely adhered to the neuronal surface, only neurons with at least 25% of their plasma membrane surrounded by IBA-1 staining were counted, as previously described (Vega-Avelaira et al., 2009). Quantitative data were recorded by an observer

blinded to the mouse genotype and the type of sample (naive, ipsilateral or contralateral to the SNI).

2.1.5. Fluorescence-Activated Cell Sorting (FACs)

For these experiments, we selected L3 and L4 DRG since they contained all somas from the common peroneal and tibial branches of the sciatic nerve (Laedermann et al., 2014), and therefore contained all neurons injured after SNI. Each assay was performed in samples with L3 and L4 DRG from 3 animals. The DRG were dissected and digested with collagenase IV (1 mg/mL, LS004188, Worthington, Lakewood, NJ, USA) and DNAse I (0.1%, LS002007, Worthington) for 1 h at 37 °C with agitation, and then with trypsin (0.25%, 15400054, Thermo Fisher Scientific, Massachusetts, USA) for 7 min at 37 °C with agitation. The digestion was neutralized with fetal bovine serum (FBS) (10%, 16000-036, Thermo Fisher Scientific) in phosphate-buffered saline (PBS). Cells were gently pipetted up and down to obtain a single cell suspension. Samples were filtered (pore size 35 μm) and the rat anti-CD32/16 antibody (1:100, 20 min, 553141; Biolegend, San Diego, CA) was used to block Fc-γRII (CD32) and Fc-γRIII (CD16) binding to IgG. Cells were incubated with antibodies recognizing the hematopoietic cell marker CD45 (1:200, 103108, clone 30-F11, Biolegend), the myeloid marker CD11b (1:100, 101227, clone M1/70, Biolegend), and the neutrophil-specific marker Ly6G (1:100, 127617, clone 1A8, Biolegend), together with a viability dye (1:1000, 65-0865-14, Thermo Fisher Scientific), for 30 min on ice. The populations of macrophages/monocytes (CD45+CD11b+Ly6G− cells) and neutrophils (CD45+CD11b+Ly6G+ cells) were determined from the markers indicated above in cells labelled with the viability dye.

Before and after incubation with the antibodies, the cells were washed three times in 2% FBS/PBS (FACS buffer). Cells were fixed with paraformaldehyde (2%, 158127, Sigma-Aldrich) for 20 min, and on the next day samples were assayed with a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). Compensation beads were used as a compensation controls, and fluorescence minus one (FMO) controls were included to determine the level of nonspecific staining and autofluorescence

associated with different cell subsets. All data were analyzed with FlowJo 2.0 software (Treestar, Ashland, OR, USA).

2.1.6. CCL2 measurement in the DRG

L3 and L4 DRG were excised and homogenized by sonication in 50 µL RIPA (0278, Sigma-Aldrich) supplemented with protease inhibitors (0.5%, P8340, Sigma-Aldrich) and phosphatase inhibitors (1%, P0044, Sigma-Aldrich). Ipsilateral or contralateral DRG from three mice were pooled, homogenized and tested as a single sample. Protein concentration in tissue homogenates was measured with the Bradford assay. The samples were stored at −80 °C until use. Twenty micrograms of protein was loaded in each well, and CCL2 levels were quantified with an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (MJE00, R&D Systems, Abingdon, United Kingdom).

2.1.7. Data analysis

For behavioral studies, statistical analyses were done with two-way repeated measures analysis of variance (ANOVA). For immunofluorescence quantification assays, statistical analyses were done with one- or two-way ANOVA, or the unpaired Student's *t* test depending on the experiment. For FACs and ELISA studies, statistical analyses were done with two-way ANOVA. The Bonferroni post hoc test was used for all ANOVA results. The differences between values were considered significant when the *P* value was less than 0.05. All data were analyzed with SigmaPlot 12.0 software (Systat Sofware Inc, San Jose, CA, USA).

2.2. Results

2.2.1. Sig-1R-KO mice show reduced mechanical allodynia after SNI

We compared the response to mechanical stimulation after SNI in WT and Sig-1R-KO mice. Both groups had similar von Frey thresholds in the hind paw before surgery (Fig. 13). After SNI, mechanical thresholds in the paw contralateral to surgery were similar to the baseline values in both Sig-1R-KO and WT animals (Fig. 13). No change was observed in pain thresholds in sham-operated control mice (data not shown). However, WT mice progressively developed mechanical allodynia in the injured limb, manifested as a significant reduction in the mechanical threshold in the paw ipsilateral to the injury starting 3 days after surgery, and peaking 7 days after SNI (Fig. 13). In contrast, Sig-1R-KO mice developed significantly less mechanical hypersensitivity compared to WT mice after SNI (Fig. 13).

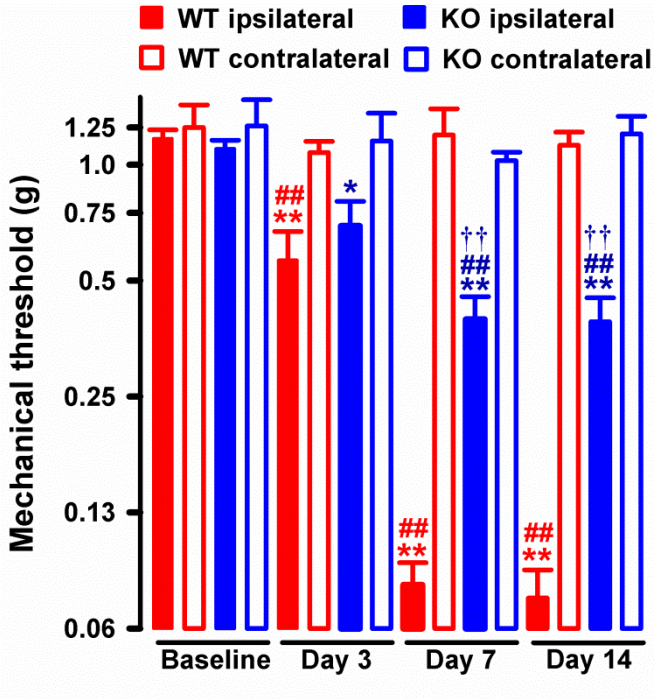

Figure 13. Comparison of spared nerve injury-induced (SNI) neuropathic mechanical allodynia in wild-type and Sig-1R-knockout mice. Von Frey thresholds were recorded 1 day before (baseline) and 3, 7 and 14 days after SNI in the paws ipsilateral and contralateral to the site of surgery. Each bar and vertical line represent the mean ± SEM of the values obtained in 8-12 animals. Statistically significant differences between the values obtained in the same paw on the day before surgery (baseline) and different days after SNI: ** P < 0.01, * P < 0.05; between ipsilateral and contralateral measurements: ## P < 0.01; and between wild-type (WT) and sigma-1 knockout (KO) groups stimulated the in paw ipsilateral to SNI: †† P < 0.01 (two-way repeated measures ANOVA followed by Bonferroni test).

Therefore, SNI induced sensory hypersensitivity in the injured limb but not in the side contralateral to the nerve injury in both WT and Sig-1R-KO mice. However, animals lacking Sig-1R developed attenuated neuropathic allodynia.

2.2.2. Sig-1R subcellular distribution in the DRG is altered in injured neurons after SNI in WT mice

To study the expression of Sig-1R in the DRG after SNI, we used immunofluorescence double labeling for NeuN and Sig-1R (Fig. 14). We found that Sig-1R was expressed by all DRG neurons (NeuN-expressing cells) from naive WT mice (Fig. 14, left panels), and that Sig-1R staining was still markedly present after SNI in DRG ipsilateral or contralateral to the surgical injury (Fig. 14, middle and right panels).

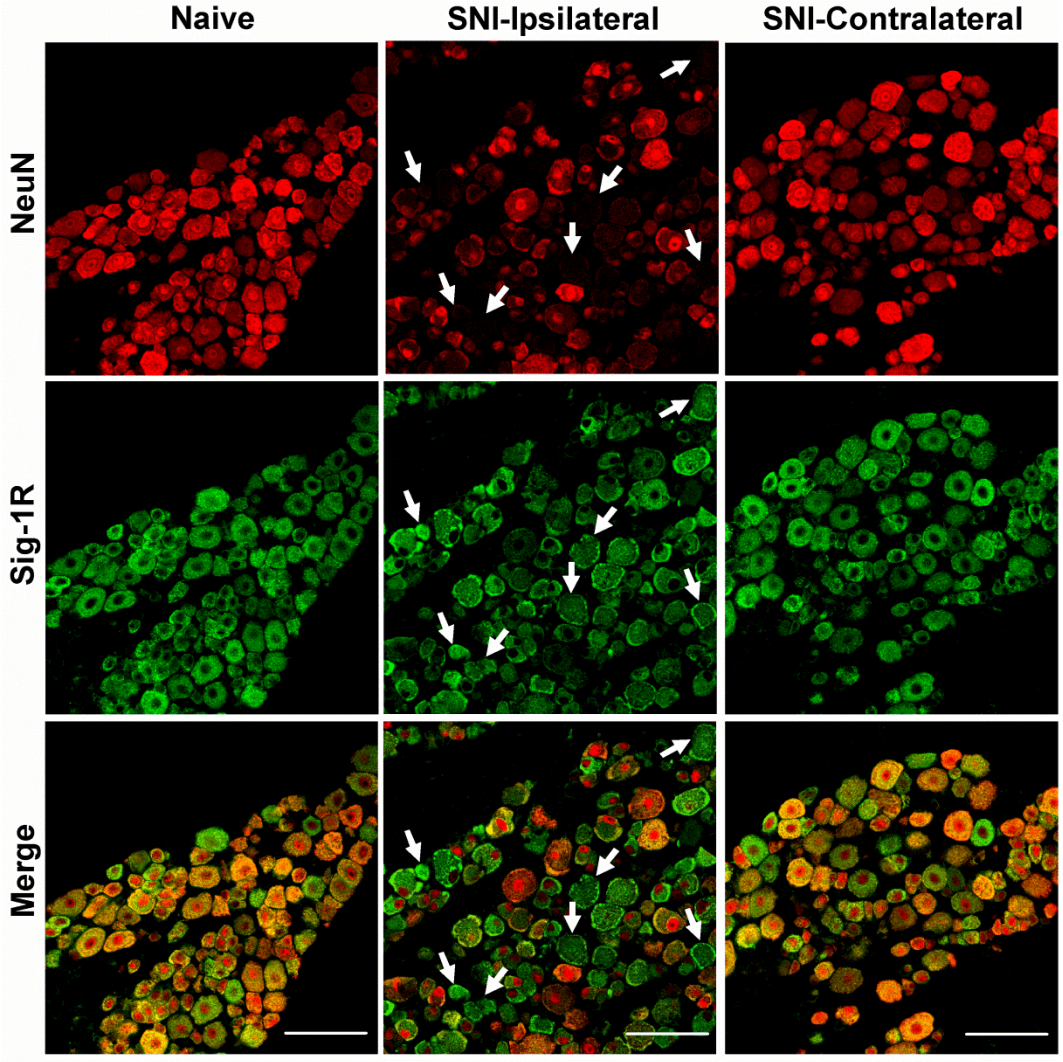

Figure 14. Sigma-1 receptor staining in the DRG persists after spared nerve injury (SNI) in wild-type mice, whereas NeuN labeling decreases ipsilateral to SNI. Representative microphotographs from double immunostaining for NeuN (red) and sigma-1 receptor (Sig-1R, green) in L4 DRG from naive mice, and ipsilateral and contralateral L4 DRG from mice with SNI 7 days after surgery. Arrows indicate neurons with decreased NeuN staining and clear Sig-1R labeling. Scale bar 100 µm.

Some cells, with a clear neuronal morphology, showed Sig1-R staining in DRG ipsilateral to the SNI but no appreciable NeuN labeling (see white arrows in Fig. 14, middle panels). Sig-1R immunolabeling was completely absent in Sig-1R-KO mice, both in naive animals and after SNI (Fig. 15), indicating the specificity of Sig-1R staining.

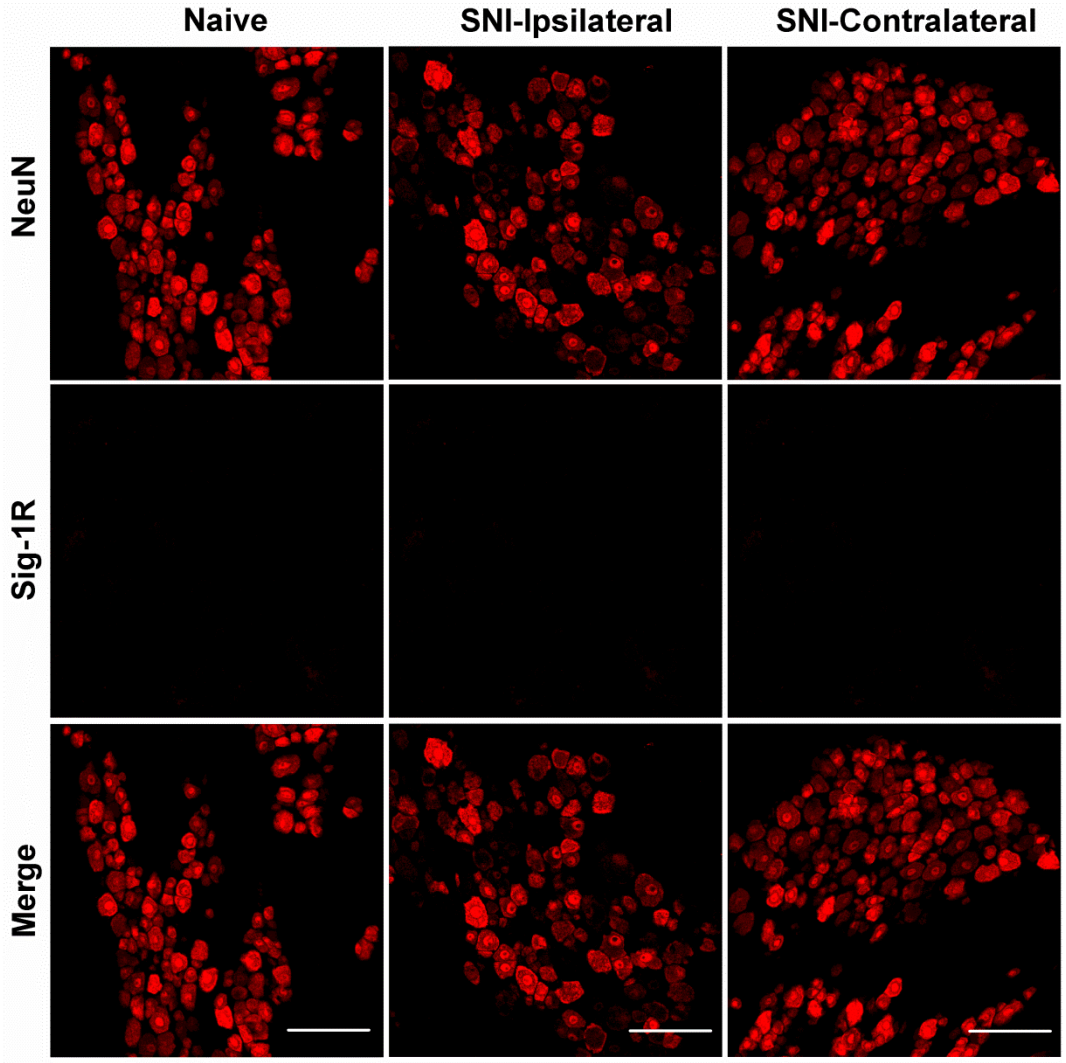

Figure 15. DRG samples from Sig-1R-KO mice are devoid of Sig-1R inmunostaining. Representative microphotographs from double immunostaining for NeuN (red) and sigma-1 receptor (Sig-1R, green) in L4 DRG from Sig-1R knockout naive mice, and ipsilateral and contralateral L4 DRG from mutant mice with spared nerve injury (SNI) 7 days after surgery. Scale bar 100 µm.

At higher magnification, variable degrees of NeuN staining intensity were evident in DRG ipsilateral to the SNI. Some neurons showed faint or no staining for cytoplasmic NeuN, although they retained NeuN labeling in the nucleus (Fig. 16, middle panels). Neurons with low levels of NeuN staining showed strongly positive labeling for the cellular stress marker ATF3, and this expression was restricted to the nuclei, whereas

neurons with intense NeuN labeling showed no ATF3 staining (Fig. 16, lower panels). Neurons in DRG from naive mice and in DRG contralateral to the SNI showed prominent NeuN staining but no ATF3 labeling (Fig. 16, left and right panels). Therefore, ATF3-expressing neurons showed less NeuN staining, and were found exclusively in injured DRG.

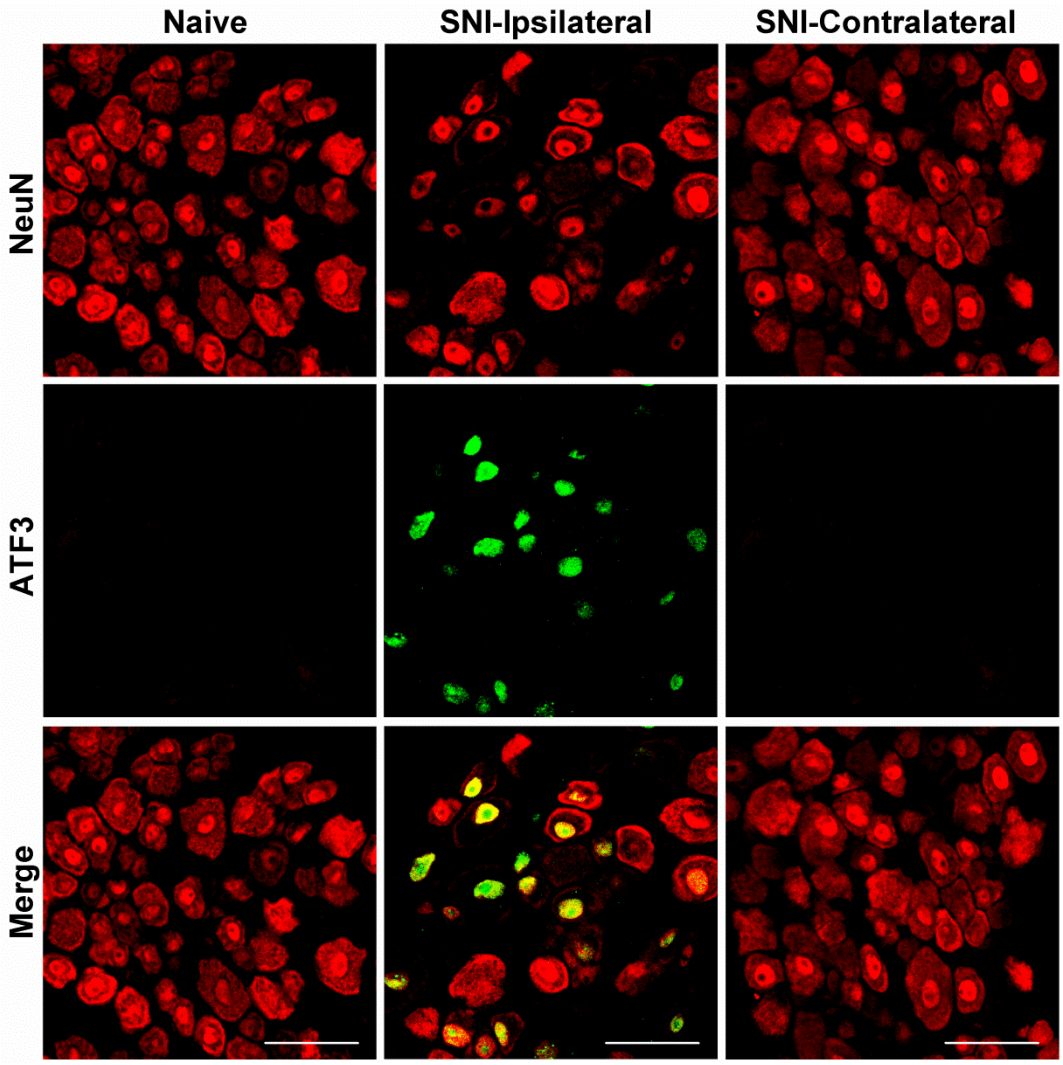

Figure 16. DRG neurons express ATF3 in wild-type mice after spared nerve injury (SNI), and show decreased NeuN staining. Representative microphotographs from double immunostaining for NeuN (red) and ATF3 (green) in L4 DRG from naive mice, and ipsilateral and contralateral L4 DRG from mice with SNI 7 days after surgery. Scale bar 50 µm.

Most Sig-1R staining was relatively homogeneous within DRG neurons from naive mice and in DRG contralateral to the injury (Fig. 17A, left and right panels), although in some neurons Sig-1R staining was concentrated at the periphery of the soma and the vicinity of the nucleus (which was devoid of Sig-1R staining) in DRG ipsilateral to the SNI (Fig. 17A, middle panels). We observed markedly more intense staining in neurons with Sig-1R translocation in DRG from the side ipsilateral to the SNI compared to DRG from naive animals and from the side contralateral to the injury (Fig. 17B). Most of the DRG neurons with evidence of Sig-1R translocation after SNI also had eccentric nuclei (Fig. 17A, middle panels), and most neurons showing Sig-1R translocation (78.4%) in the DRG ipsilateral to the SNI also expressed ATF3 in the cell nucleus (Fig. 17A, middle panels and Fig. 17C).

In summary, SNI induced nuclear eccentricity, a decrease in NeuN staining, and ATF3 expression in neurons from injured DRG. These changes were accompanied by a change in the subcellular distribution of Sig-1R in sensory neurons, i.e. translocation to the periphery of the soma and the vicinity of the cell nucleus.

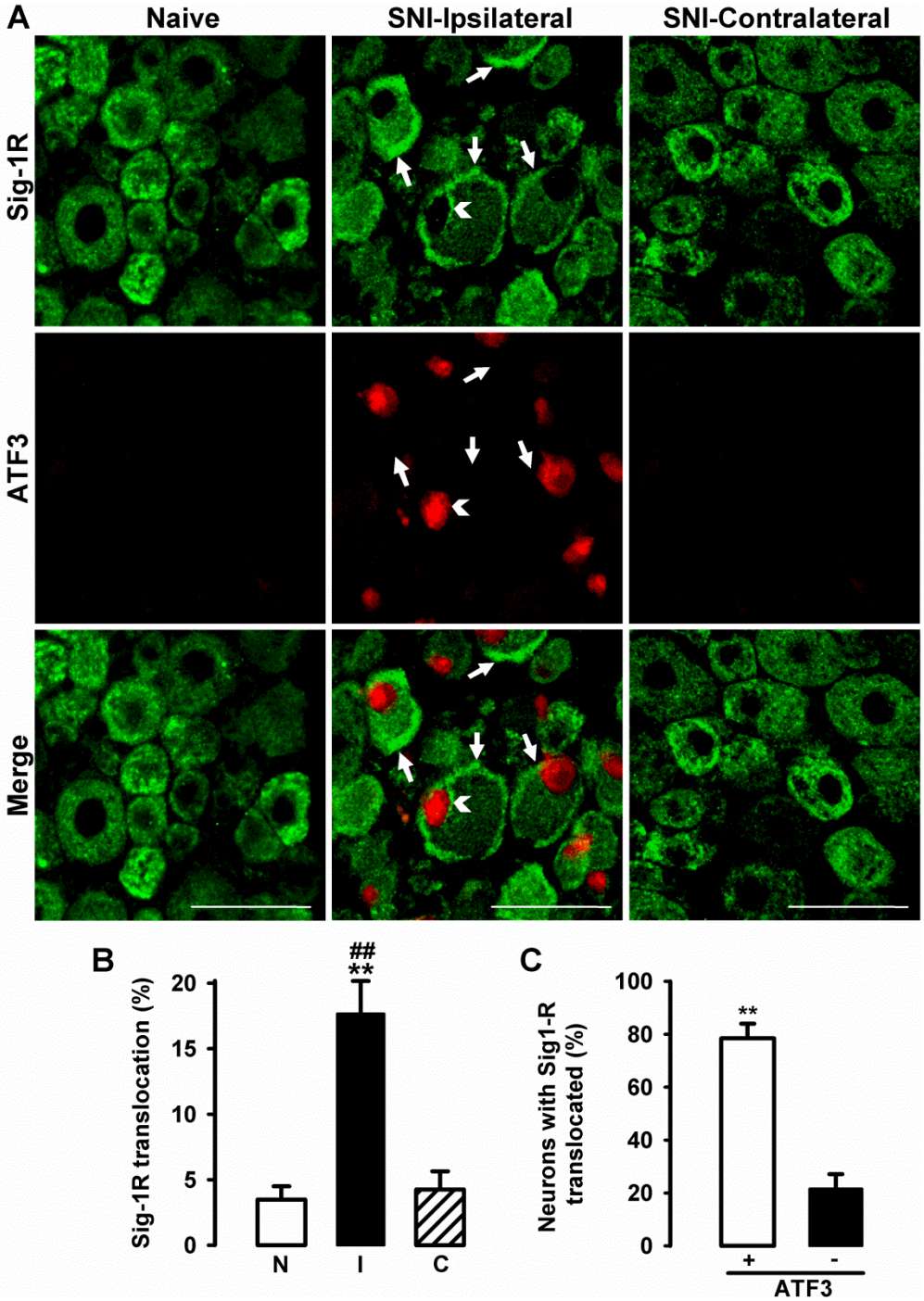

Figure 17. Subcellular distribution of sigma-1 receptor in DRG after spared nerve injury (SNI) in wild-type mice. A) Representative microphotographs of double labeling for sigma-1 receptors (Sig-1R, green) and ATF3 (red) in L4 DRG from naive mice, and ipsilateral and contralateral L4 DRG from mice with SNI 7 days after surgery. Arrows indicate neurons with Sig-1R staining concentrated at the periphery of the soma. Arrowheads indicate neurons with Sig-1R concentrated in close vicinity to the cell nucleus. Scale bar 50 µm. (B) Quantification of neurons showing Sig-1R translocation in samples from naive animals, and in DRG ipsilateral and contralateral to SNI. N: naive, I: ipsilateral; C: contralateral. Statistically significant differences between naive and ipsilateral groups: $^{**}P < 0.01$; and between the values in the ipsilateral and contralateral groups: $^{\#\#} P < 0.01$ (one-way ANOVA followed by Bonferroni test). (C) Quantification of the percentage of neurons with Sig-1R translocation that did or did not express ATF3. Statistically significant differences between the values in ATF3+ and ATF3− neurons with translocated Sig-1R: $^{**}P < 0.01$ (Student's *t* test). Each bar and vertical line represent the mean ± SEM of the values obtained in 4 animals in both graphs.

2.2.3. Macrophage/monocyte infiltration in the DRG after SNI is modulated by Sig-1R

Because of the relationship between macrophage/monocyte infiltration into the DRG and neuropathic pain (see Introduction for references), we carried out immunofluorescence double labeling with Sig-1R and the macrophage/monocyte marker IBA-1 in WT mice. Figure 18A shows representative images for Sig-1R and IBA-1 staining in DRG from naive animals, injured animals, and samples from the side contralateral to the injury. In naive animals little or no appreciable IBA-1 staining was detected (Fig. 18A, left panels). However, after SNI, macrophages/monocytes clustered around the cell bodies in Sig-1R-stained neurons, forming ring-like structures in the DRG ipsilateral to the injury. This finding suggested a close neuron-immune cell interaction (Fig. 18A, middle panels). However, few or no IBA-1+ cells were detected in DRG contralateral to the nerve injury (Fig. 18A, right panels). We observed a minimal overlapping between Sig-1R and IBA-1 labeling in the regions where the neuron and macrophage/monocyte are closest (Fig. 18A, middle panels), supporting the close neuron-immune cell interaction, and suggesting that Sig-1R was expressed in sensory neurons but not in macrophages or monocytes. The characteristic ring-like IBA-1 labeling was seen mostly in neurons with Sig-1R staining at the periphery of the soma (Fig. 18A middle panels). In fact, a majority (69.5%) of neurons with Sig-1R translocation to the periphery of the soma were surrounded by macrophages/monocytes, whereas these immune cells rarely accumulated around neurons that did not show evidence of Sig-1R translocation: only 20.5% of neurons without translocation were surrounded by IBA-1+ cells (Fig. 18B). These results suggested that Sig-1R might be involved in the communication between neurons and macrophages/monocytes.

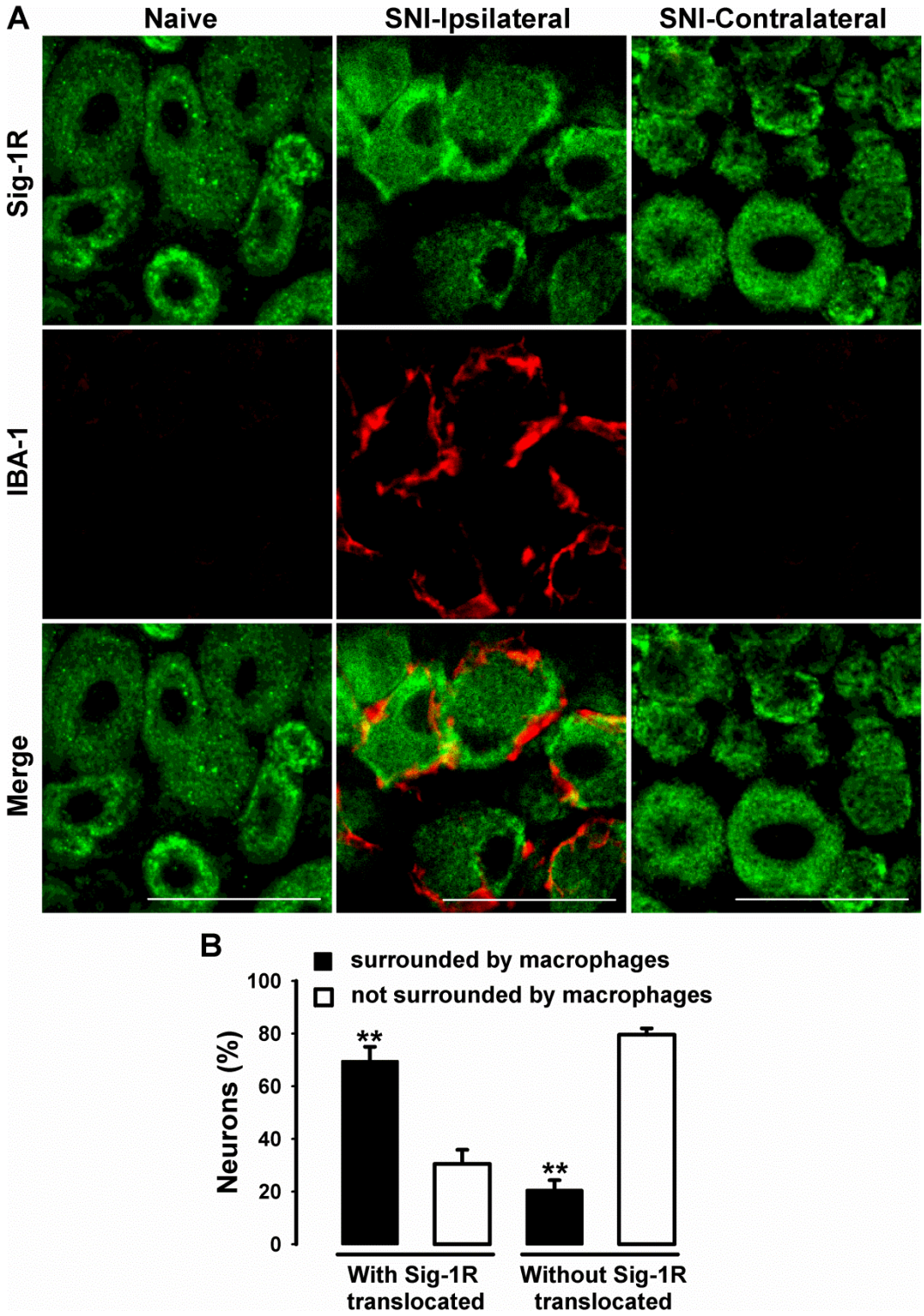

Figure 18. DRG neurons with sigma-1 receptor translocation are surrounded by macrophages/monocytes forming ring-like structures after spared nerve injury (SNI). (A) Representative microphotographs from double immunostaining for sigma-1 receptor (Sig-1R, green) and IBA-1 (red) in L4 DRG from naive mice, and ipsilateral and contralateral L4 DRG from mice with SNI 7 days after surgery. Scale bar 50 µm. (B) Quantification of the percentage of neurons with and without Sig-R1 translocation, and surrounded by macrophages or not. Each bar and vertical line represent the mean ± SEM of the values obtained in 4 animals. Statistically significant differences between the values in the groups surrounded and not surrounded by macrophages: ** $P < 0.01$ (two-way ANOVA followed by Bonferroni test).

We then compared macrophage/monocyte infiltration into DRG in WT and Sig-1R-KO mice after SNI. Figure 19 shows representative images for the neuronal marker NeuN and IBA-1+ macrophages/monocytes in DRG from non-injured WT and Sig-1R-KO mice, and from samples obtained 7 days after SNI in both genotypes (Fig. 19A). In naive WT and Sig-1R-KO animals, little or no appreciable IBA-1 staining was detected (Fig. 19A, left panels). However, after SNI, macrophages/monocytes clustered around the cell bodies of neurons with ring-like structures in DRG ipsilateral to the injury in WT mice, and these neurons often showed decreased NeuN labeling (white arrows in Fig. 19A, middle panels). IBA1+ cells were still present in Sig-1R-KO animals, but at much lower levels than in WT mice (Fig. 19A, middle panels). The quantification of IBA-1+ cells confirmed the considerable infiltration of macrophages/monocytes into ipsilateral DRG in both WT and Sig-1R-KO mice after SNI (compared to samples from naive animals and samples from the side contralateral to the injury), and also showed that the increase in macrophage/monocyte numbers in the DRG was smaller in Sig-1R-KO animals (Fig. 19B).

We also quantified macrophage/monocyte infiltration in DRG from injured and non-injured mice by FACS with different markers (CD45+CD11b+Ly6G− cells), and investigated the possible infiltration of neutrophils (CD45+CD11b+Ly6G+) into the DRG after SNI. We found a markedly greater presence of macrophages/monocytes in injured DRG from WT mice compared to naive DRG and to samples from the side contralateral to the injury (Fig. 19C). Sig-1R-KO mice also showed a significantly larger number of macrophages/monocytes in injured DRG compared to non-injured DRG, although this increase was significantly less pronounced than in samples from WT mice (Fig. 19C). We did not find a significant infiltration of neutrophils in the injured DRG compared to non-injured DRG from either WT or Sig-1R-KO mice (data not shown). These results are evidence that SNI induced massive macrophage/monocyte infiltration into the DRG, and that infiltration was markedly weaker in mice that lacked Sig-1R. Accordingly, these findings suggest that Sig-1R plays an important role in macrophage/monocyte recruitment after nerve injury.

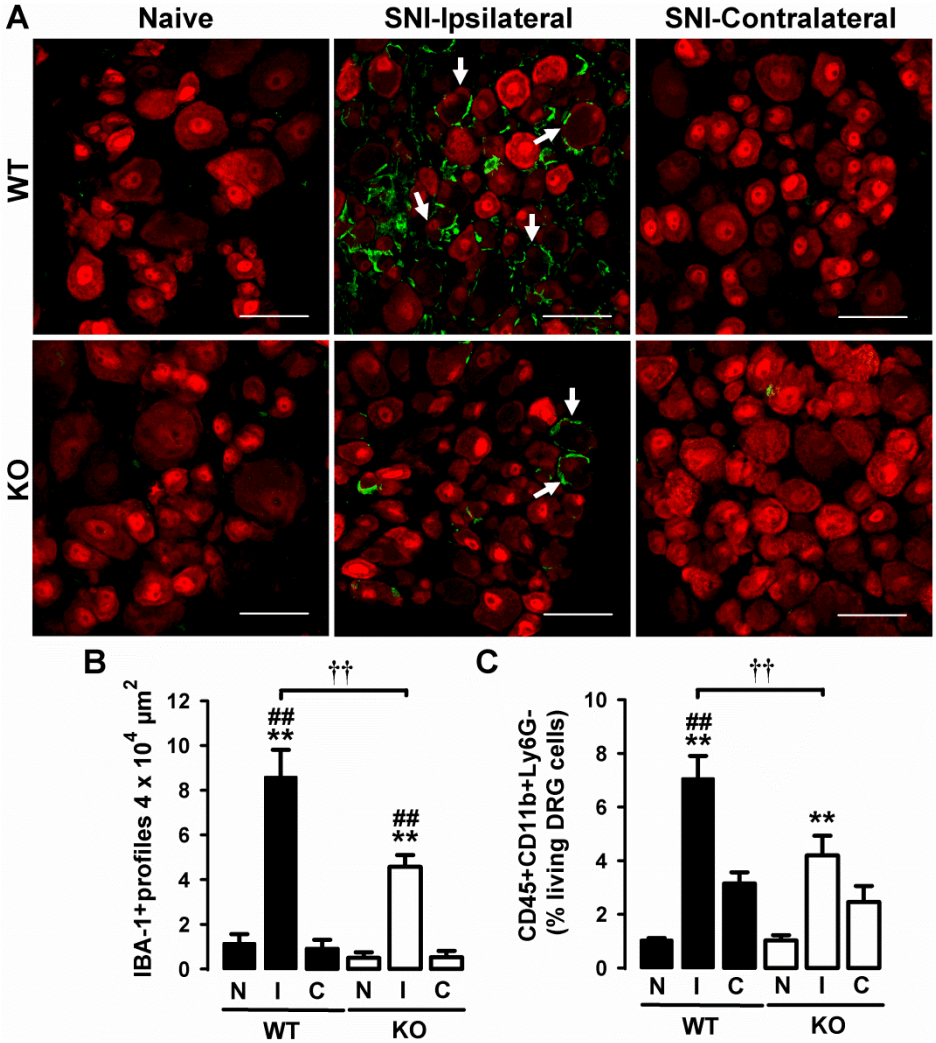

Figure 19. Sigma-1 receptor knockout mice show less macrophage/monocyte infiltration than wild-type mice in the DRG after spared nerve injury (SNI). (A) Representative microphotographs of double immunostaining for IBA-1 (green) and NeuN (red) in L4 DRG from naive mice, and ipsilateral and contralateral L4 DRG from mice with SNI 7 days after surgery. Experiments were performed in wild-type (WT) mice and Sig-1R knockout mice (KO). Scale bar 50 µm. (B) Quantification of the number of macrophages/monocytes (IBA1+ profiles) per 4×10^4 µm^2. Each bar and vertical line represent the mean ± SEM of the values obtained in 3-6 animals. N: naive, I: ipsilateral, C: contralateral. (C) CD45+CD11b+Ly6G− cells as determined by FACS. Each bar and vertical line represent the mean ± SEM of the values obtained in 4 or 5 batches of DRG. Each batch was obtained with L3 and L4 DRG from 3 animals. (B and C) Statistically significant differences between the values in naive and SNI ipsilateral groups: ** $P < 0.01$; between ipsilateral and contralateral groups of mice with the same genotype: ## $P < 0.01$; and between DRG ipsilateral to SNI in the WT and KO groups: †† $P < 0.01$ (two-way ANOVA followed by Bonferroni test).

2.2.4. Sig-1R-KO mice show lower levels of CCL2 in the DRG after SNI than WT mice

The findings presented in the preceding section indicated that Sig-1R might be relevant for macrophage/monocyte recruitment, so we then measured the levels of chemokine CCL2 in DRG from non-injured WT and Sig-1R-KO mice, as well as at several time-points after SNI in samples from both genotypes. Non-injured mice from both the WT and Sig-1R-KO groups had similar baseline levels of CCL2. The concentration of CCL2 in injured DRG from WT mice showed a bell-shaped time-course, increasing as early as 24 h after surgery, peaking on day 3 after the injury, and subsequently decreasing on day 7 (Fig. 20). Sig-1R-KO mice also had increased levels of CCL2 in DRG ipsilateral to the SNI, with a time-course similar to that in WT mice, but the increase in CCL2 content was significantly smaller that in WT mice (Fig. 20). The increase in CCL2 was restricted to injured DRG, given that CCL2 concentration did not change at any time-point tested in the DRG contralateral to the injury in either in WT or Sig-1R-KO mice. These results suggest that Sig-1R modulated CCL2 release in the DRG after SNI.

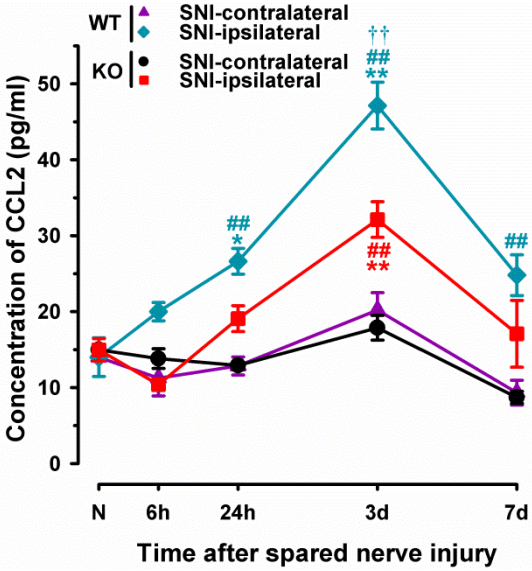

Figure 20. Sigma-1 receptor knockout mice show lower CCL2 levels in DRG after spared nerve injury (SNI) than wild-type mice. CCL2 levels in L3 and L4 DRG ipsilateral and contralateral to SNI in wild-type (WT) and Sig-1R knockout (KO) mice were measured by ELISA. Each point and vertical line represents the mean ± SEM of the values obtained in 3-5 batches of DRG samples for each time point. Each batch was obtained with L3 and L4 DRG from 3 animals. Statistically significant differences between the values from DRG samples from naive and SNI mice: * $P < 0.05$, ** $P < 0.01$; between the values in DRG samples ipsilateral and contralateral to SNI within the same genotype: ## $P < 0.01$; and between the values in DRG ipsilateral to SNI in the WT and KO groups: †† $P < 0.01$ (two-way ANOVA followed by Bonferroni test).

2.3. Discussion

We show that Sig-1R-KO mice develop less mechanical allodynia than WT mice in response to peripheral nerve injury. Spared nerve injury was followed by the presence of eccentric nuclei, a decrease in NeuN staining, and ATF3 expression in neurons from injured DRG. These changes were accompanied by Sig-1R translocation to the periphery of the soma and the vicinity of the cell nucleus in peripheral sensory neurons. In addition, we found an increase in the levels of CCL2, and in macrophage/monocyte numbers, in injured DRG. These immune cells formed ring-like structures mainly in neurons in which Sig-1R was translocated. In Sig-1R-KO mice the level of CCL2 and macrophage/monocyte infiltration into the DRG were both lower after nerve injury than in WT mice.

Peripheral nerve injuries such as nerve transection that takes place during surgical procedures or as a consequence of other trauma lead to neuropathic pain in a considerable number of patients (Borsook et al., 2013; Hewson et al., 2018). Here we show that mechanical allodynia in the intact sural branch territory of mice, after transection of the common peroneal and tibial branches of the sciatic nerve (i.e. SNI), developed progressively in WT animals, with a time-course similar to that reported in previous studies based on mouse models (Bourquin et al., 2006; Cobos et al., 2018; Bravo-Caparrós et al., 2019). We show that Sig-1R-KO mice developed significantly less mechanical allodynia than WT mice after SNI. This result is in agreement with our recent study in the same neuropathic pain model (Bravo-Caparrós et al., 2019), and with previous reports that mutant animals lacking Sig-1R developed less sensory hypersensitivity than WT mice in other neuropathic pain models (de la Puente et al., 2009; Nieto et al., 2012; Nieto et al., 2014; Wang et al., 2018).

We found that Sig-1R immunoreactivity in normal conditions was expressed in the somas of all peripheral sensory neurons from the mouse DRG. The receptor was located in the cytoplasm but not inside the cell nucleus, as previously reported (Mavlyutov et al., 2016; Montilla-García et al., 2018). We show that Sig-1R staining in sensory neurons changed dramatically after nerve injury only in DRG from the side ipsilateral to SNI, where it accumulated in the periphery of the soma and in close proximity to the cell nucleus. We demonstrated the specificity of the antibody used for

Sig-1R staining by verifying the absence of immunoreactivity in samples from Sig-1R-KO mice that were sham operated and after SNI. It was previously reported that when Sig-1R is activated in cell cultures by either sigma-1 agonism or cellular stress, it translocates from its intracellular location to the plasmalemmal area within the extended endoplasmic reticulum (or to the plasma membrane itself) and to the nuclear envelope (Su et al., 2010; Tsai et al., 2015). Therefore, the alterations we observed in Sig-1R labeling induced by SNI might indicate that nerve injury triggered Sig-1R activation. To our knowledge, this is the first experimental evidence that changes in the subcellular distribution of Sig-1R are identifiable in animal tissues under pathological conditions, and not only in cell cultures.

We show that some DRG neurons from the side ipsilateral to SNI underwent other changes in addition to the alterations in subcellular Sig-1R distribution, including nuclear eccentricity, which has long been known to be due to severe neuronal damage (Matthews and Raisman, 1972; Johnson and Sears, 2013), and a marked decrease in NeuN staining. To our knowledge there are no previously published studies in DRG tissues that document the decrease in NeuN labeling after nerve injury. However, it is well known that staining for this protein decreases markedly after axotomy in several nervous system structures, at both central and peripheral levels (e.g. Collombet et al., 2006; Duan et al., 2016). Therefore, the decrease in NeuN staining we observed in the DRG may reflect changes in injured neurons. In this connection, the decrease in NeuN staining was accompanied by a clear increase in ATF3 labeling in the neuronal nucleus - a finding of relevance given that ATF3 is considered among the most reliable markers of injury in DRG neurons (Tsujino et al., 2000; Laedermann et al. 2014). Importantly, we observed that the majority of DRG neurons with Sig-1R translocation also expressed ATF3, suggesting that Sig-R1 activation (translocation) was mostly restricted to injured neurons.

It is known that nerve injury induces immune cell infiltration into the affected DRG (Scholz and Woolf, 2007). We found a marked increase in macrophages/monocytes in the DRG after SNI, in agreement with previous studies (Cobos et al., 2018; Kwon et al., 2013). We did not observe increased neutrophil recruitment in the DRG after nerve injury, which is also in agreement with previous reports (Lindborg et al., 2018). We

show that macrophages/monocytes accumulated and formed ring-like structures mostly in sensory neurons where Sig-1R was translocated. It was previously reported that these ring-like structures are seen preferentially in injured neurons (Vega-Avelaira et al., 2009), a finding consistent with the Sig-1R translocation we report here in injured neurons. The accumulation of macrophages/monocytes around neurons with translocated Sig-1R may indicate that Sig-1R activation is important for the interaction between these immune cells and injured sensory neurons. In support of this hypothesis, we show here that in Sig-1R-KO mice, macrophage/monocyte infiltration into the DRG after nerve injury was much weaker than in WT mice. It is worth noting that under our experimental conditions, macrophages/monocytes did not express Sig-1R, given that labeling for the receptor in DRG samples was restricted to sensory neurons and was not seen in IBA-1+ cells. Therefore, the modulation by Sig-1R of macrophage/monocyte infiltration in the DRG after nerve injury is unlikely to be attributable to direct sigma-1-mediated effects on these immune cells, but rather can be explained by curtailed communication between neurons and macrophages/monocytes.

The communication between peripheral sensory neurons and immune cells is complex, but currently the role of CCL2 in this interaction is unquestioned. This chemokine has potent chemotactic activity and is involved in macrophage recruitment in the DRG after nerve injury (Zigmond and Echevarria, 2019). In this connection, it is well known that injured neurons are the main source of this chemokine in DRG after nerve injury (e.g. Zhu et al., 2014; Kwon et al., 2015; Liu et al., 2016). In fact, CCL2 blockade results in a decrease in the number of macrophages/monocytes that migrate into the DRG after nerve injury (e.g. Niemi et al., 2013; Lindborg et al., 2018). Thus CCL2 is one of the main molecules that mediate macrophage/monocyte recruitment by injured neurons in the DRG. Here we show that CCL2 was produced soon after nerve injury, with a bell-shaped time-course. The levels of CCL2 increased as early as 1 day after injury, reached a maximum at 3 days, but subsequently decreased 7 days after the injury. This time-course of CCL2 production after peripheral nerve injury is consistent with at least one previous report in another mouse model (Kwon et al., 2015). According to the present findings, Sig-1R-KO mice had reduced levels of this

chemokine in the injured DRG after SNI, and this lower activity may partly account for the decreased macrophage/monocyte infiltration in the DRG in our mutant animals after nerve injury.

Macrophage and monocyte recruitment into the DRG plays a pivotal role in neuropathic pain, because they promote a proinflammatory environment in the affected ganglia which can in turn sensitize non-injured neurons, and thus contribute to the development of sensory hypersensitivity (Raoof et al. 2018; Cobos et al., 2018). This mechanism would explain, at least in part, the tactile allodynia seen in the intact sural branch territory after SNI. In addition, CCL2 has direct effects on sensory neurons in that it increases their excitability (Wang et al., 2010; White et al., 2005). Therefore, both the decrease in CCL2 levels and the reduced macrophage/monocyte infiltration into the DRG after nerve injury in Sig-1R-KO mice may contribute to the lower sensory hypersensitivity we observed in these mutant animals.

It was previously reported that Sig-1R antagonism is also able to decrease microglia recruitment and astrocyte activation in the dorsal horn during pathological pain conditions, and this might contribute to the known ameliorative effects of Sig-1R inhibition on sensory hypersensitivity (Zhu et al., 2015; Jeong et al., 2015; Choi et al., 2016). It is interesting to note that CCL2 released from primary afferents into the spinal cord also participates in microglia recruitment and astrocyte activation in the spinal dorsal horn (van Steenwinckel et al., 2011; Zhu et al., 2014). Therefore, in light of the present results, it can be hypothesized that at least part of the previously described central effects induced by Sig-1R inhibition during pain conditions may be attributable to the actions of this receptor on peripheral sensory neurons, rather than to its direct central effects.

In summary, we found that nerve injury triggered the activation of Sig-1R in peripheral sensory neurons, and that this receptor played a key role in the recruitment of macrophages/monocytes into the injured DRG, and were thus directly involved in a process that is crucial for the development of neuropathic pain. Therefore, Sig-1R inhibition may be a potentially effective approach to alleviating neuropathic pain not

only because of its previously described central effects, but also because of its clear, simultaneous actions at the peripheral level.

CONCLUSIONS

CONCLUSIONS

A. Specific conclusions

1. Wild-type mice develop prominent neuropathic pain after SNI, which is manifested by cold (acetone test), mechanical (von Frey test) and heat (Hargreaves test) hypersensitivity. In contrast, after SNI, the Sig-1R-KO mice do not develop cold allodynia and show a marked inhibition of mechanical allodynia, although they develop heat hyperalgesia as WT mice.

2. Systemic acute administration of the selective Sig-1R antagonist S1RA inhibits all three types of SNI-induced hypersensitivity in WT mice. The ameliorative effects of S1RA are reversed by the administration of the Sig-1R agonist PRE-084 and are absent in Sig-1R-KO mice, indicating the selectivity of S1RA-induced effects.

3. The opioid antagonist naloxone and its peripherally restricted analog naloxone methiodide prevent S1RA-induced acute ameliorative effects in mechanical and heat hypersensitivity, but not in cold allodynia, indicating that opioid-dependent and -independent mechanisms are involved in the effects of this Sig-1R antagonist.

4. Repeated treatment with S1RA (twice a day during 10 days) reduces SNI-induced cold, mechanical and heat hypersensitivity without inducing analgesic tolerance during treatment. These effects are observed up to 12 h after the last administration, when S1RA is undetectable in plasma or brain, indicating long-lasting pharmacodynamic effects.

5. The Sig-1R is present in every single neuron of the dorsal root ganglia and is distributed homogeneously throughout the cytoplasm in control conditions. After SNI the Sig-1R translocates to the periphery of the soma and the vicinity of the nucleus, mostly in injured ($ATF3^+$) neurons.

6. WT mice produce the chemokine CCL2 in the DRG ipsilateral to the SNI, and this is followed by a massive infiltration of the DRG by macrophages/monocytes, which mainly cluster around sensory neurons with the Sig-1R translocated. In contrast, Sig-1R-KO mice show reduced levels of CCL2 and a decreased infiltration of macrophages/monocytes into the DRG ipsilateral to the SNI, which suggest an important role of peripheral Sig-1R on the sensory neuron-macrophage/monocyte communication in the DRG after peripheral nerve injury.

B. General conclusions

1. Sigma-1 receptor plays a pivotal role in neuropathic pain induced by peripheral nerve transection in mice and at least part of this effect is mediated peripherally, through an enhancement of endogenous opioid effects and an inhibition of sensory neuron-macrophage/monocyte communication in the DRG.

2. Pharmacological antagonism of Sig-1R may have therapeutic value for the treatment of neuropathic pain induced by the transection of peripheral nerves.

ABBREVIATIONS

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AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA: Analysis of variance

ASIC: Acid-Sensing Ion Channel

ASIC1a: Acid-sensing ion channel of the 1a subtype

ATF3: Activating Transcription Factor 3

ATP: Adenosine Triphosphate

BD-1047:N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino)ethylamine dihydrobromide

BD-1063: 1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride

BDNF: Brain-Derived Neurotrophic Factor

Ca^{2+}: Calcium

CCD: Chronic Compression of Dorsal root ganglia

CCI: Chronic Constriction Injury

CCL2: Chemokine ligand 2

CCLs: Chemokine Ligands

CCR2: C-C Chemokine Receptor 2

CD11b: Cluster of differentiation molecule 11B

CD45: Cluster of differentiation 45

CD45R/B220: Cluster of differentiation 45R/B cell isoform of 220

CGRP: Calcitonin Gene-Related Peptide

CIPN: Chemotherapy-Induced Peripheral Neuropathy

CL^-: Chlorine

CNS: Central Nervous System

CX3CL1: C-X3-C Motif Chemokine Ligand 1 (Fractalkine)

CX3CR1: C-X3-C Motif Chemokine Receptor 1

DRG: Dorsal Root Ganglia

ELISA: Enzyme-Linked Immunosorbent Assay

EOP: Endogenous Opioid Peptide

ER: Endoplasmic Reticulum

ERK: Extracellular signal-Regulated Kinase

FACS: Fluorescence-Activated Cell Sorting

FBS: Fetal Bovine Serum

FMO: Fluorescence Minus One

GABA: Gamma-Aminobutyric Acid

GDNF: Glial cell line-Derived Neurotrophic Factor

GPCR: G-Protein-Coupled Receptors

HPLC-MS/MS: High-Performance Liquid Chromatography-triple quadrupole Mass Spectrometry

i.p.: intraperitoreal

IASP: International Association for the Study of Pain

Iba-1: Ionized calcium-Binding Adapter molecule 1

IL: Interleukin

K^+: Potassium

l-THP: levo-tetrahydropalmatine

Ly6G: Lymphocyte antigen 6 complex locus G6D

M1: Macrophage subtype 1

M2: Macrophage subtype 2

MOR: μ-opioid receptor

MR200: [(+)-methyl (1R,2S)-2-{[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]methyl}-1-phenylcyclopropanecarboxylate]

Na^+: Sodium

NaV: Voltage-gated sodium

NE-100: 4-methoxy-3-(2-phenylethoxy)-N,N-dipropylbenzeneethanamine

NeuN: Neuronal Nuclei

NGF: Nerve Growth Factor

NMDA: N-Methyl-D-Aspartate

NMDAR: N-Methyl-D-Aspartate Receptor

NMIN: N-(2-morpholin-4-yl-ethyl)-2-(1-naphthyloxy) acetamide

NR1: subunit 1 of NMDAR

NR2B: subunit 2B of NMDAR

P2R: Purinergic Receptor 2

P2RX4: Purinergic Receptor P2X 4

P2RX7: Purinergic Receptor P2X 7

p38 MAPK: p38 Mitogen-Activated Protein Kinase

PBS: Phosphate-Buffered Saline

PKA: Protein Kinase A

PKC: Protein Kinase C

PM: Plasma Membrane

PNS: Peripheral Nervous System

PRE-084: 2-[4-morpholinethyl]1-phenylcyclohexanecarboxylate hydrochloride

PSNL: Partial Sciatic Nerve Ligation

ROS: Reactive Oxygen Species

RT: Room Temperature

s.c.: subcutaneous

S1RA: 4-[2-[[5-methyl-1-(2-naphtalenyl)1H-pyraol-3-yl]oxy]ethyl]

SCI: Spinal cord Contusion Injury

SEM: Standard Error of the Mean

Sig-1R: Sigma-1 Receptor

Sig-1R-KO: Sigma-1 Receptor knockout

SNI: Spared Nerve Injury

TCRβ: T cell receptor beta chain

TLR: Toll-Like Receptor

TNF-α: Tumor Necrosis Factor-alpha

TRP: Transient Receptor Potential

TRPA1: Transient Receptor Potential Ankyrin 1

TRPM8: Transient Receptor Potential Melastatin 8

TRPV1: Transient Receptor Potential Vanilloid 1

WT: Wild-Ype

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PUBLISHED ARTICLES

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1. Articles directly relacted to this doctoral thesis

Bravo-Caparrós I, Perazzoli G, Yeste S, Cikes D, Baeyens JM, Cobos EJ, Nieto FR. 2019. Sigma-1 receptor inhibition reduces neuropathic pain induced by partial sciatic nerve transection in mice by opioid-dependent and -independent mechanisms. Front Pharmacol 10:613.

Bravo-Caparrós I, Perazzoli G, Cronin SJ, Ruiz-Cantero MC, Vela JM, Mohamed FH, Penninger JM, Baeyens JM, Cobos EJ, Nieto FR. 2019. Sigma-1 receptors control neuropathic pain and macrophage infiltration into the dorsal root ganglion after peripheral nerve injury. Submitted.

2. Other articles

Bravo-Caparrós I, Nieto FR. 2017. Roles for CD8+ T cells and IL-10 in the resolution of paclitaxel-induced neuropathic pain. J Neurosci 37(11):2803-2805.

Cobos EJ, Nickerson CA, Gao F, Chandran V, **Bravo-Caparrós I**, González-Cano R, Riva P, Andrews NA, Latremoliere A, Seehus CR, Perazzoli G, Nieto FR, Joller N, Painter MW, Ma CHE, Omura T, Chesler EJ, Geschwind DH, Coppola G, Rangachari M, Woolf CJ, Costigan M. 2018. Mechanistic differences in neuropathic pain modalities revealed by correlating behavior with global expression profiling. Cell Rep 22(5):1301-1312.

Tejada MÁ, Montilla-García Á, González-Cano R, **Bravo-Caparrós I**, Ruiz-Cantero MC, Nieto FR, Cobos EJ. 2018. Targeting immune-driven opioid analgesia by sigma-1 receptors: Opening the door to novel perspectives for the analgesic use of sigma-1 antagonists. Pharmacol Res 131:224-230.

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Jiménez-López J[1], **Bravo-Caparrós I[1]**, Cabeza L, Nieto FR, Ortiz R, Perazzoli G, Fernández-Segura E, Cañizares FJ, Baeyens JM, Melguizo C, Prados J. 2019. Nanoformulation of large pegylated cationic liposomes loaded with paclitaxel improves antitumor effect in lung cancer and prevents painful neuropathy. J Control Release. Submitted.

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